UNIVERSITY of TASMANIA

BIOPROCESS MONITORING USING SEQUENTIAL INJECTION CAPILLARY ELECTROPHORESIS

By

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Declaration

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Statement of co-authorship

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

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Ala A. Alhusban was the primary author (70%) and collected all the papers, organized the manuscript and wrote the review. The co-authors contributed a total of 30% to the published work. Michael Breadmore and Rosanne Guijt contributed to idea, its structure and reviewing the manuscript.

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4. Alhusban, A. A., Breadmore, M. C., Gueven, N., Guijt, R. M. **Parallel Monitoring of Pharmacological Assays using Sequential Injection Capillary Electrophoresis**, *Analytical Chemistry*, ac-2015-04822f, xxx-xxx. **(Chapter 4)**

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8. Alhusban, A. A., Breadmore, M. C., Gueven, N., Guijt, R. M. **Online Monitoring of Cell Culture by Sequential Injection Capillary Electrophoresis**. 40th International Symposium on High-Performance Liquid-Phase Separations and Related Techniques, Hobart, Australia, 18th to 21th November 2013.

9. Alhusban, A. A., Breadmore, M. C., Gueven, N., Guijt, R. M. **On-line Monitoring of Cell Cultures.** The 3rd International Conference

Implementation of Microreactor in Biotechnology (IMTB), Opatija, Croatia 10th to 13th May 2015.

10. R.M. Guijt, A. A. Alhusban, A. Shallan, U. Kalsoom,, M. Boyce, M.C. Breadmore, **Alternative Approaches for Sample Preparation in Capillary Electrophoresis**, CECE 2015 – International Interdisciplinary Meeting on Bioanalysis, Brno, Czech Republic, 21th to 23th September 2015

List of Abbreviations

BGE	Background electrolyte
CAPS	Cleaved Amplified
CGE	Capillary Gel Electrophoresis
cITP	Capillary isotachophoresis
CTAB	Cetyl trimethylammonium bromide
DAD	Diode array detector
DTT	Dithiothreitol
ED	Electrochemical detection
EDTA	Ethylenediaminetetraacetic acid
ESI-IT-MS	Electrospray ion-trap mass spectrometry
ESI-TOF-MS	Time-of-flight mass spectrometry
HBLC	High Performance Liquid Chromatography
HDMB	Hexadimethrine bromide
hGF	Human growth factor
HPMC	Hydroxypropyl methylcellulose
HTAB	Hexadecyltrimethylammonium bromide
LA-coA	Lactate-coenzyme A
LDH	Lactate dehydrogenase
LIF	Laser Induced Fluorescence
MEKC	Micellar electrokinetic
MES	2-(N-Morpholine) ethanesulfonic acid

MOA	Mars Organic Analyser
NADP	Nicotinamide adenine dinucleotide phosphate
PAGE	Polyacrylamide gel electrophoresis
PDCA	Pyridine-2,6-dicarboxylic acid
PCR	Polymerase Chain Reaction
PLA	Poly Lactic Acid
RQ	Respiration Quotient
SCFA	Short Chain Fatty Acids
SDS	Sodium dodecyl sulfate
SHMT	Serine hydroxymethyltransferase
SSCP	Single-stranded conformation polymorphism
Tris	Tris(hydroxymethyl)aminomethane
TTAB	Tetradecyltrimethyl ammonium bromide

Abstract

Bioprocess monitoring has received significant interest over the past few years. The production of biopharmaceuticals synthesized by living cells during fermentation or cell culture processes is a rapidly growing field. Additionally, cell based assays have replaced many *in vivo* assays because of ethical and regulatory restrictions on working with laboratory animals. Biological processes are naturally susceptible to variability because living cells consume substrates and produce metabolites and products in a dynamic way with variations in metabolic rate across short time intervals. For the production of biopharmaceuticals, the FDA recommends documentation of nutrient and metabolite time profiles in the process analytical technology (PAT) policy to ensure product quality. At present, the majority of cell culture based monitoring is restricted to a few end point based assays that do not reflect the dynamic metabolic processes in cells that influence the final product. Therefore, a detailed and continuous monitoring of the bioprocesses in each production batch would significantly help manufacturers to control product quality, increase production yields and reduce production costs. At the same time, online monitoring of bioprocesses will also significantly enhance our understanding of fundamental dynamic cellular metabolic reactions that cannot be easily ascertained by end point measurements, and in turn facilitate pharmacological and biotechnological studies employed for screening and compounds testing.

This thesis explores the potential of capillary electrophoresis (CE) for bioprocess monitoring. CE is a powerful and high resolution separation technique with the potential to provide detailed chemical information quickly using small sample volumes. First, the potential of Sequential injection capillary electrophoresis (SI-CE) for monitoring lactate production, an important metabolic indicator, during adherent

mammalian cell culture, was examined. A new sampling interface was developed to sample from the medium covering a culture of human embryonic kidney cell line HEK293 and mouse fibroblast cell lines. Changes in lactate concentration in the cell culture medium were measured every 20 minutes over 3 days, requiring only 8.73 μL of sample per analysis. Second, a SI-CE system was developed for automated, on-line, near real-time monitoring of suspension cultures by integrating microfluidic components for cell counting and analyte extraction with the high-resolution separation technique. The correlation of cell growth of a human lymphocyte cell line with changes in the essential metabolic markers including glucose, glutamine, leucine/isoleucine and lactate provided new insights in the metabolic changes over time. Using only 8.1 mL of media (41 μL per analysis), the metabolic status and cell density were recorded every 30 minutes over 4 days. This system provides a promising new solution to meet the future demands in process monitoring in the biopharmaceutical industry.

The developed platform for monitoring suspension cultures was extended to simultaneous monitoring of five parallel suspension cultures, capable of conducting cell density measurement and a high-resolution separation every 12 minutes for 4 days. This system was applied to study the metabolic effects of the drugs rotenone, β-lapachone and clioquinol on metabolism using lactate as indicator. For each drug, suspension culture experiments for three drug concentrations and two controls were monitored in parallel. Over the 4 days, 5.78 mL of media was consumed from each culture, equating to 60 μL per analysis. The fully automated system offers high sample throughput, good temporal resolution and low sample consumption combined with robustness, sensitivity and flexibility which provides a promising new platform for pharmacological and biotechnological studies.

1. Table of Contents

Preface

The importance of bioprocess monitoring

A bioprocess is a biological process where living cells and/or their components are studied or utilized to produce a desired product, with fermenters and cell cultures applied throughout life sciences. Moreover, biopharmaceuticals produced from living cells during fermentations or cell cultures is a fast growing field. Around 35 percent of the authorized therapeutic products are biopharmaceuticals. The significance of cell culture based technologies is in their employment in drug screening and discovery as well as the pharmacodynamics studies of the existing and the new potential drug targets. Therefore, it is essential to monitor such bioprocesses to ensure the safety of the product together with satisfying economic and regulatory demands, and to provide a feasible ways to perform accurate and reliable pharmacological and biotechnological studies.

Cellular bioprocesses are mainly controlled through the manipulation of their external environment, which are the cell culture medium and its chemical composition. Currently, most of the chemical analysis in bioprocesses does not reflect the dynamic metabolic processes in cells that influence the final product because these assays are restricted to end point analysis. New systems for continuous and detailed monitoring of the bioprocess would aid manufacturers to control product quality. Furthermore, it would enhance the understanding of fundamental dynamic cellular metabolic reactions and will reduce production costs and increase production yields. The same technology would also provide the data required for deeper understanding in cell based assays increasingly used to substitute *in vivo* assays as a result of restrictions on working with laboratory animals.

Capillary electrophoresis and its potential for bioprocess monitoring

At present, diverse methods are employed for bioprocess monitoring, the most important being enzymatic assays, biosensors, spectroscopic techniques, such as UV, FTIR or RAMAN, and analytical separations such as liquid chromatography (LC), mass spectrometry, and capillary electrophoresis (CE). Enzymatic assays are specific and simple, but time consuming – normally needing hours per analysis, are limited to a single analyte and may require expensive reagents. Biosensors are specific and selective but are again limited to one analyte and require regular calibrations. Spectroscopic techniques have the capability to provide detailed chemical information, but despite advances in the interpretation of spectra, resolving complex analyte sets with similar functional groups remains challenging. Chromatographic techniques are sensitive, versatile and highly reproducible. Yet, separations are slow (typically tens of minutes) and sample pre-treatment is typically required. Mass spectrometry can been used to detect a whole set of analytes in cell culture media, but the extensive sample clean-up limits its use for automated continuous monitoring. Additionally, it is unable to resolve compounds with similar mass, complicating the resolution of mono- and polysaccharides.

CE is a powerful technique characterized by quicker separations compared to LC and has been broadly used to separate a range of analytes, from small ions to macromolecules in various fields. For bioprocess monitoring, CE has been employed for the analysis of discrete samples, using commercially available single or multiple capillary instruments. It has similarly been employed for on-line monitoring of a range of analytes in various matrices and environments, but only a few reports present dedicated, online sampling interfaces for CE analysis. The low sample volume, ability to be automated, robustness, speed, high sample throughput, good temporal

resolution, selectivity, flexibility and multi-analytes analysis makes CE ideally suited for monitoring cell cultures and cell-based assays.

Project aims and scopes of study

As pointed out above, bioprocess monitoring is highly desirable. Increasingly stringent demands on the production of biopharmaceuticals demand monitoring of process parameters that impact on their quality. And with a move away from animal models, early stage pharmacological studies depend on *in vitro* methodologies for screening and testing compounds. The advantages of CE mentioned early make it an attractive analytical technique to reach this goal provided suitable sampling methods are available. The overall aim of this thesis is to develop new sampling interfaces to enable to capitalise on the potential of CE as instrumental analytical tool for bioprocess monitoring. The findings will provide insights towards realistic involving of CE for the employment in the field of bioprocess monitoring.

The specific aims of the project are to:

- Develop a CE method and sampling interface for monitoring lactic acid production in adherent cell cultures.
- Develop a CE method and microfluidic components to expand the monitoring to more substrates and metabolites in suspension cell cultures and correlating them to cell density.
- Develop a CE method for simultaneous monitoring of multiple suspension cell cultures with correlation to cell density.
- Develop a CE method for parallel monitoring of pharmacological assays.

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Alhusban, A. A., Breadmore, M. C.; Guijt, R. M. 2013, Capillary electrophoresis for monitoring bioprocesses, Electrophoresis, 34(11), 1465-1482.

Chapter 2

On-line Sequential Injection-Capillary Electrophoresis for near real-time Monitoring of Extracellular Lactate in Cell Culture Flasks[2]

2.1 Introduction

Cell culture of eukaryotic cells is widely applied throughout life sciences. At the small scale, cell based assays have replaced many *in vivo* assays because of ethical and regulatory restrictions on working with laboratory animals. Additionally, the production of biopharmaceuticals synthesized by living cells during fermentation or cell culture processes is a rapidly growing field. According to the FDA, approximately 30–40 percent of the authorized medical products in 2012 were biopharmaceuticals. Bioprocessing offers many advantages. For example, the production of vaccines by cell culture technology instead of conventional methods provides the capability for rapid manufacturing start-up in case of a pandemic because characterized cell lines can be stored and are thus readily available. Moreover, the risk of impurities can be reduced because vaccine production takes place in a highly controlled, closed and sterile environment[1]. Cell culture based technologies are intensively employed in drug discovery[2], and are significant tools for drug screening and new potential drug targets studies[3]. In 2012 alone, 35 novel biologics were developed by the biopharmaceutical industry and approved by the FDA[4]. In this context it is important to note

[2] Update from an article published in, **Alhusban, A. A.; Gaudry, A. J.; Breadmore, M. C.; Gueven, N.; Guijt, R. M., On-line sequential injection-capillary electrophoresis for near-real-time monitoring of extracellular lactate in cell culture flasks.** *Journal of Chromatography A* **2014, 1323, 157-162.**

that bioprocess monitoring in the production process of biopharmaceuticals is essential to ensure the safety of the product as well as to satisfy economic and regulatory demands.

At present, the majority of cell culture based monitoring is restricted to a few end point based assays that do not reflect the dynamic metabolic processes in cells that influence the final product. Therefore, a detailed and continuous monitoring of the bioprocesses in each production batch would significantly help manufacturers to control product quality, increase production yields and reduce production costs[5]. At the same time, online monitoring of bioprocesses will also significantly enhance our understanding of fundamental dynamic cellular metabolic reactions that cannot be easily ascertained by end point measurements. Lactate is one of the major products of eukaryotic and prokaryotic cells and is one of the most important organic acids in extracellular media that can be used to monitor cellular metabolism and energy status. It is produced from glucose and glutamine in mammalian cells[6]. It is important to monitor because it affects the physicochemical stability of the bioprocess medium by reducing pH levels and is outright toxic to some cells. In addition, under certain conditions, cultured cells can use lactate as an alternative source for carbon, even in the presence of glucose[7]. At the same time, lactate can be used as an indicator of biological activity. For example, metabolic shifting from lactate production to lactate consumption was reported to result in improvements of process performance regarding productivity, scalability, process robustness and cell growth[8].

Due to the tight connection between extracellular and intracellular metabolic pathways, cellular bioprocesses are primarily controlled by manipulating the external environment in form of the composition of the cell culture medium[9]. Therefore, timely information about even small changes in the concentration of extracellular lactic acid during cell culture will directly help to control and improve the efficiency of the bioprocess. Realizing this depends on the availability of suitable and functional tools that can be used for monitoring[10].

Currently, a number of different methods are used for lactate detection, the most important being enzymatic assays[11,12], and analytical separations such as liquid chromatography (LC)[13,14] and capillary electrophoresis (CE)[15,16]. Enzymatic assays are highly specific, instrumentally simple, but sometimes too time consuming – typically requiring hours per measurement[17], and are restricted to a single analyte. Chromatographic techniques are sensitive, versatile, and have excellent reproducibility. However, separations are slow and typically sample pre-treatment is required. Capillary Electrophoresis (CE) is a powerful alternative characterized by faster separation compared to LC and has been widely used to separate a diverse range of analytes, from small ions through to macromolecules in many fields[18,19], in particular for monitoring bioprocesses. As discussed in a recent review, CE has been readily employed for the analysis of discrete samples, utilizing commercially available single or multiple capillary instruments[20]. It has also been employed for on line monitoring of a variety of analytes in different matrices and environments, but only a few reports present dedicated, online sampling interfaces for CE analysis[20]. One of those, a CE method with conductivity detection was applied for automated continuous on-line analysis of 23 ions in tap water over two days[21]. CE was also used for on-line determination of perchlorate in biological samples such as breast milk, human urine, serum, red wine and cow's milk using a supported liquid membrane[22]. A filter probe was integrated with a computerized pneumatic sampling system to monitor the bioaccumulation of Cu^{2+}, Zn^{2+}, Co^{2+} and Cd^{2+} in the bacteria species *Rhodococcus* sp.[23].

Another CE method using LIF detection was successfully employed for microbial analysis of water collected from two local streams through continuous electrokinetic injection under field amplified conditions[24].

The low sample volume makes CE ideally suited for monitoring cell culture conditions and cell-based assays. In this study, an automated, robust and portable SI-CE setup was

developed by modifying the experimental set up previously reported by Blanco *et al.*[25]. A flow-through interface was designed to sample cell-free media for monitoring lactate production by the human embryonic kidney cell line HEK293 and mouse fibroblast cell lines (Fe18.83 and Fe20.4). The unique combination of the sampling interface and SI-CE system minimised sample consumption and analysis time. Using this system, 72 samples were analysed per day using less than 700 µL of sample per day, enabling monitoring of lactate production by HEK293 cells *in vitro* over three days, using less than 10% of the total media volume. The system was also applied to study the difference in lactate production between a WT and mitochondrial function compromised ND4 knockout mouse fibroblast cell lines.

2.2 Materials and methods

2.2.1 Chemicals

All reagents were analytical grade reagent obtained from Sigma–Aldrich (Sydney, AUS) and were used as supplied unless otherwise stated. Solutions were prepared in Milli-Q water (Millipore, Bedford, MA, USA). Lactate standard solution (10 mM) was prepared weekly and stored at 8 °C by dissolution of its sodium salt. Chloride standard solution (2 M) was prepared monthly from sodium chloride and stored at room temperature.

The cationic polyelectrolyte poly(ethylenimine) (PEI) (ACROS organics, Geel, Belgium) was added to BGE. A polyelectrolyte multilayer coated fused-silica capillary prepared from hexadimethrine bromide (HDMB) (Sigma–Aldrich, St. Louis, MO, USA) and poly(sodium 4-styrene sulfonate homopolymer) (PSS) and (HDMB) again in order to reverse the EOF. Cetyltrimethylammonium bromide (CTAB) was added to BGE in case of dynamic coating. Two studied BGEs including a combination of (tris(hydroxymethyl)aminomethane (Tris))/Ncyclohexyl-2-aminoethanesulfonic acid (CHES), pH 8.85 and 2-(N-

morpholino)ethanesulfonic acid (MES)/20 mM l-histidine (His), pH 6.15 were evaluated for optimum separation.

2.2.2 Instrument design and operation

A scheme of the instrumental set up used for this work is shown in Figure 2-1. The SI-CE instrumentation is composed of two peristaltic pumps (PeriWaves, CorSolutions, Ithaca, NY, USA) for sample and BGE delivery to the system. A two-position injector valve (MXP-7980, Rheodyne, Oak Harbor, WA, USA) was used to direct sample or BGE to the analytical system. A PEEK T-piece-connector (P-727, Upchurch Scientific, OakHarbor, WA, USA) was used for interfacing the flow system and the CE capillary. This interface allows inserting the capillary with a small internal volume (0.57 μL).

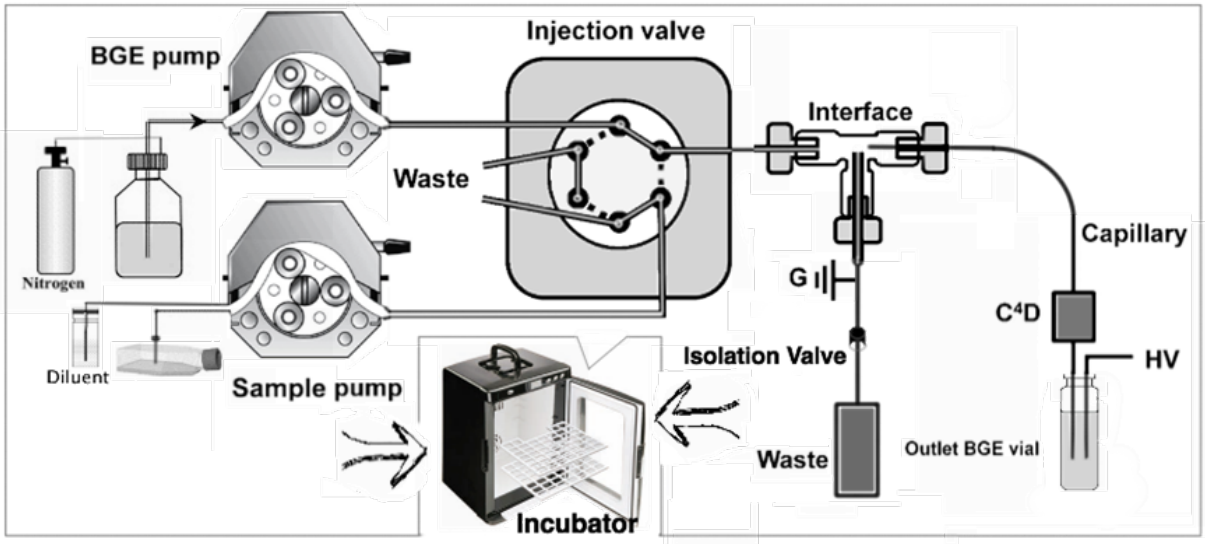


Figure 2-1 Schematic diagram of the SI-CE system placed inside an incubator. Sample and BGE and introduced by the peristaltic sample and BGE pumps, respectively. An injector valve directs the BGE or sample towards the sampling interface. When the isolation valve is open, sample or BGE flows into the waste vial, when the valve is closed, the liquid will be forced into the 50 μm I.D. fused silica capillary. The CE separation is achieved by applying +30 kV (HV) to the outlet vial while the interface was grounded (G). A capacitively coupled contactless conductivity detector (C^4D) was positioned 10 cm from the outlet of the 85 cm long capillary.

All connecting tubing was selected based on a narrow diameter (381 μm) to reduce dead volume and to minimize turbulent flow. The capillary inlet was fixed near the interface centre at a position to eliminate consecutive sample carry over, and the outlet end was immersed in a 25 mL glass vial filled with BGE. A stainless steel syringe needle was cut to yield a 2 cm long, 0.51 mm internal diameter tube and was employed as electrode and connected to the interface through the waste tubing. A solenoid (isolation) valve (HP225K021, NResearch, West Caldwell, NJ, USA) was linked on the waste tubing at the T piece outlet to control solutions direction either to capillary or to waste. A commercial capacitively coupled contactless conductivity detector, C^4D (TraceDec), was supplied by Innovative Sensor Technologies (Strassahof, Austria). Detection parameters were optimized according to capillary internal diameter. The detector operational parameters were selected as following: frequency, 2× high (5 MHz); voltage, 18 db; gain, 200%; off set, 008. The detector sensing head was fixed 10 cm from the outlet end of the CE capillary. A high-voltage power supply was used for CE under reversed polarity, with the anode (+) electrode immersed in the outlet glass vial. The injector valve, pumps and high-voltage power supply were connected with an NI USB-6212 data acquisition interface board (National instruments, Austin, TX, USA) and controlled using Lab-View v8.1 (National Instruments).

It is important to note that all the described components were sufficiently compact to be placed inside an incubator (BBD6220, Heraeus, Thermo-Fisher, Kendo Laboratory Products, Zurich, Switzerland) that was operated at fixed humidity (90%) and constant temperature (37 $^{\circ}$C). A CO_2 Laser Engraver (40 W CO_2 Deluxe Hobby Laser, Full Spectrum Laser LLC, Las Vegas, NV, USA) was used to fabricate a hole in both the sampling tubing interface and cell culture flask. Sequential injection of samples was optimized and the sequence of analysis steps is detailed in Table 2-1.

Table 2-1 Sequence of events in the SI-CE system operation, the HV power supply is only activated during separation

	Setup operation	Position of valve	Used BGE volume µL	Used sample volume µL	Flow rate ($\mu L min^{-1}$)	Used time (s)	Isolation Valve	Sample pump	BGE Pump
1	***Flushing of capillary and interface***	**1**	**100**	**0**	**100**	**60**	**Close**	**Off**	**On**
2	***Equilibrate***	**1**	**0**	**0**	**0**	**1**	**Open**	**Off**	**Off**
3	***Sample introduction***	**2**	**0**	**8.33**	**25**	**20**	**Open**	**On**	**Off**
4	***Sample injection***	**2**	**0**	**0.33**	**20**	**1**	**Close**	**On**	**Off**
5	***Flushing of interface***	**1**	**83.33**	**0**	**500**	**10**	**Open**	**Off**	**On**
6	***Separation***	**1**	**923.33**	**0**	**50**	**1108**	**Open**	**Off**	**On**

The SI-CE system was operated as follows: the capillary and the interface were filled and flushed with BGE in order to equilibrate the capillary and to remove any air bubbles. The sample was introduced into the interface before being injected into the capillary by briefly closing the solenoid valve. The interface was then flushed with BGE (83 µL) at a high flow rate (0.5 $mL.min^{-1}$) before applying the high voltage (+30 kV) for electrophoretic separation.

2.2.3 Electrophoretic conditions

Fused-silica capillaries (50 µm I.D; Polymicro Technologies, Phoenix, AZ, USA) with 85 cm length (75 cm effective length) were used. Capillaries were conditioned by flushing with 1 M NaOH at 0.5 $\mu L.min^{-1}$ for 5 min then Milli-Q water at the same rate for 5 min. For the multilayer polymer coating, the capillary was flushed with 1% aqueous solution of HDMB for 5 min at 0.5 $\mu L.min^{-1}$, 1% PSS for 5 min at 0.5 $\mu L.min^{-1}$, 1% aqueous solution of HDMB for 5 min at 0.5 $\mu L.min^{-1}$ and finally with BGE for 30 min. The BGE solutions after optimization consisted of 50 mM/50 mM of Tris (tris(hydroxymethyl)-aminomethane) and CHES (cyclohexyl-2-aminoethanesulfonic acid) at pH 8.85 and 20 mM/20 mM 2-(N-morpholino)ethanesulfonic acid (MES) and $_L$-histidine (His) at pH 6.15. 0.02% (w/v) PEI was added in order to stabilize the coating and to alter the selectivity. For the CTAB dynamic coating, after flushing with 1 M NaOH and Milli-Q water, capillaries were flushed for 60 min with BGE containing 0.03 mM CTAB. Two BGEs were evaluated: Tris/CHES and MES/HIS. Separation was performed at −30 kV. The BGE bottle in case of using Tris/CHES was connected to a nitrogen line to keep the solution and the headspace with fresh nitrogen to prevent alteration of the pH from CO_2 absorption. The EOF in the electropherogram appeared as a negative peak from with the low-conductivity sample.

2.2.4 Application in monitoring mammalian cell culture

2.2.4.1 Human embryonic kidney cells

Human embryonic kidney cells (HEK293, Sigma–Aldrich, Castle Hill, Australia) were routinely cultured under standard conditions (5% CO_2, 37 °C, 90% humidity) in Dulbeccos Modified Eagles Medium (DMEM, InVitro, Noble Park North, Australia) supplemented with 10% fetal calf serum (FCS, VWR, Murarrie, Australia) and penicillin/streptomycin/glutamine (Invitrogen, Mulgrave, Australia). For measurement of lactate production, cells were seeded at 8×10^5 cells/cm^2 in growth media in specially prepared T75 flask (Corning® 75 cm^2 rectangular canted neck cell culture flask with vented cap) with a 510 μm I.D. hole at the top cover which was engraved using a laser engraver (at 30% cut speed, 5% laser power and 5 repeats) and sealed to prevent any contamination prior to cultivation. The flask also contained the sampling interface, which was inserted through the hole at its top cover and was prepared by engraving a 500 μm I.D. hole 500 μm from the end of 508 μm I.D. tubing (1522, Upchurch Scientific fluorinated ethylenepropylene) using a laser engraver (26 repeats at 100% speed, 5% laser power). Cells were left to adhere over night before they were washed with PBS twice and the medium was changed to a total of 20 mL Leibowitz L15 medium (VWR, Murarrie, Australia) containing 5% FCS and penicillin/streptomycin/glutamine. Cells were immediately transferred to a second humidified, cell culture incubator (90% humidity, 37 °C, without CO_2 supply) that contained the sampling and measurement set up. The incubator was not opened for the duration of the experiment.

2.2.4.2 Mouse fibroblast cells

Mouse fibroblast cells (Fe18.83, ND4 knockout, David Thorburne, Melbourne, Australia) and (Fe20.4, Wild Type WT, Thorburne, Melbourne, Australia) were routinely cultured under standard conditions (5% CO_2, 37 °C, 90% humidity) DMEM supplemented with 5% fetal calf

serum and penicillin/streptomycin/glutamine. For measurement of lactate production, cells were seeded at 2×10^5 cells/cm^2 in growth media in the particularly prepared T75 flask following the procedure described in the previous section. After the first three-day monitoring experiment, media was removed and the cells adhered at the bottom of the T75 flask were washed twice with PBS before 20 mL fresh Leibowitz L15 medium containing 5% FCS and penicillin/streptomycin/glutamine was added. The flask was directly transferred back to the cell culture incubator (without CO_2 supply) and connected with the analytical system (the connection tubing was rinsed at least 3 times with 70% ethanol and autoclaved to eliminate any source of contamination) to start the second 3-day monitoring experiment.

2.3 Results and discussion

2.3.1 Method development

2.3.1.1 SI-CE of lactate

A CE method for monitoring lactate in cell cultures must be a fast, efficient, selective, and automated. Most importantly, it should only use minute amounts of sample per analysis. As our previously described SI-CE system[21,25] had the potential to meet the sampling requirements, this set-up was modified to develop a suitable separation method. The most significant change compared to our previous work is the use of peristaltic pumps instead of the MilliGAT piston pumps. Peristaltic pumps were selected because the liquid solution does not come in contact with the pump, hence eliminating the risk of blocking/damaging valuable parts of the pump. Cell culture broths contain proteins, lipids and cellular debris, which can likely adhere to and contaminate and destruct the inside of other pump types, while in peristaltic pump the tubing can be simply replaced. The accuracy of the employed pumps was tested at the used flow rate and the deviation from the programmed flow was found to be approximately 1.5%. The peristaltic pumps are programed to deliver BGE and sample,

respectively. First, the interface and the capillary are flushed with BGE, closing the solenoid isolation valve to flush BGE through the capillary. Subsequently, the six-port valve is switched to allow the sample pump to introduce sufficient sample to fill the interface (solenoid isolation valve open) and to inject a small plug of sample onto the capillary by the build-up of pressure when the solenoid isolation valve is closed for 1 s. Then the six-port vale is switched again to pump BGE into the system to remove excess sample from the interface and to start separation (solenoid isolation valve open). To reduce BGE and sample consumption, the sample pump is stopped when the system is flushed with BGE and vice versa. Both dynamic and permanent coatings were explored for finding the best electrophoretic separation conditions in terms of reproducibility, repeatability, linearity, coating and base line stabilities, limit of detection and limit of quantification.

As CTAB has been used as effective EOF reversal agent in C^4D of anions[26], this was initially examined[27,28]. Using MES/HIS and operating the system at a constant 37 °C inside the incubator, the precision of lactate electrophoretic mobilities (7.14% RSD) was comparable to those obtained at room temperature (8.87% RSD), but lower baseline stability was noticed which led to decreased precision in the lactate peak area. Changing to Tris/CHES enhanced the electrophoretic mobility precision (3.92% RSD) and the base line stability, but the precision of the peak areas was still poor (15.19% RSD). These results suggested that the CTAB dynamic coating was not sufficiently reliable and a polyelectrolyte multilayer coating was adopted using the polymers HDMB and PSS. A 3-layer, HDMB/PSS/HDMB coating was prepared, which significantly improved the repeatability as demonstrated in Table 2-2 with excellent electrophoretic mobility precision (0.07% RSD) and good base line stability and very good precision of the peak areas (3.98% RSD). Because the baseline when using Tris/CHES BGE was slightly more stable than when using MES/HIS, this BGE was selected for further optimization.

Table 2-2 Summary of validation results of lactate monitoring developed methods in terms of repeatability, reproducibility, linearity and limit of detection and quantification.

	CTAB added to BGE MES/HIS	**CTAB added to BGE Tris/CHES**	**HDMB/PSS/HDMB, PEI added to BGE MES/HIS**	**HDMB/PSS/ HDMB, PEI added to BGE Tris/CHES**
Intraday precision (Electrophoretic mobilities) n= 5	**7.14%**	**3.92%**	**0.11%**	**0.07%**
Interday precision (Electrophoretic mobilities), n=5	**8.23%**	**4.35%**	**0.15%**	**0.09%**
Intraday precision (Peak areas), n=5	**15.09%**	**15.19%**	**10.22%**	**3.98%**
Interday precision (Peak areas), n=5	**18.41%**	**22.14%**	**11.36%**	**6.57%**
LOD	**2 µM**	**3 µM**	**3 µM**	**3 µM**
LOQ	**1 µM**	**13 µM**	**15 µM**	**11 µM**
Base line stabilities	+	++	+++	++++
Linearity (0.15-5 mM)	**0.9589**	**0.9622**	**0.9954**	**0.9977**

2.3.1.2 SI-CE of lactate in cell culture media

Cell culture media contains a wide variety of compounds including inorganic ions, amino acids, sugars, vitamins and proteins. It was essential to further optimize the developed method to resolve lactate from interference from other anions. The PEI concentration, BGE concentration and pH, and capillary coating, I.D. and length were all investigated. The optimized conditions were as follows: 85 cm × 50 μm I.D. (10 cm to detector) HDMB/PSS/HDMB coated capillary, 25 mM Tris/35 mM CHES, pH 8.65 with 0.02% PEI. Figure 2-2 shows an electropherogram of cell culture media spiked with 0.125 mM lactate and without added lactate.

To test the monitoring system, a lactate standard solution (3.5 mM) was monitored at 20 min intervals over 2 days using the optimized conditions. Figure 2-3 illustrates the change in peak area over time, the oscillating variation is most likely related to the pulsation of the peristaltic pump, introducing different amounts of sample into the capillary depending on the phase of the pulse and the closing of the isolation valve.

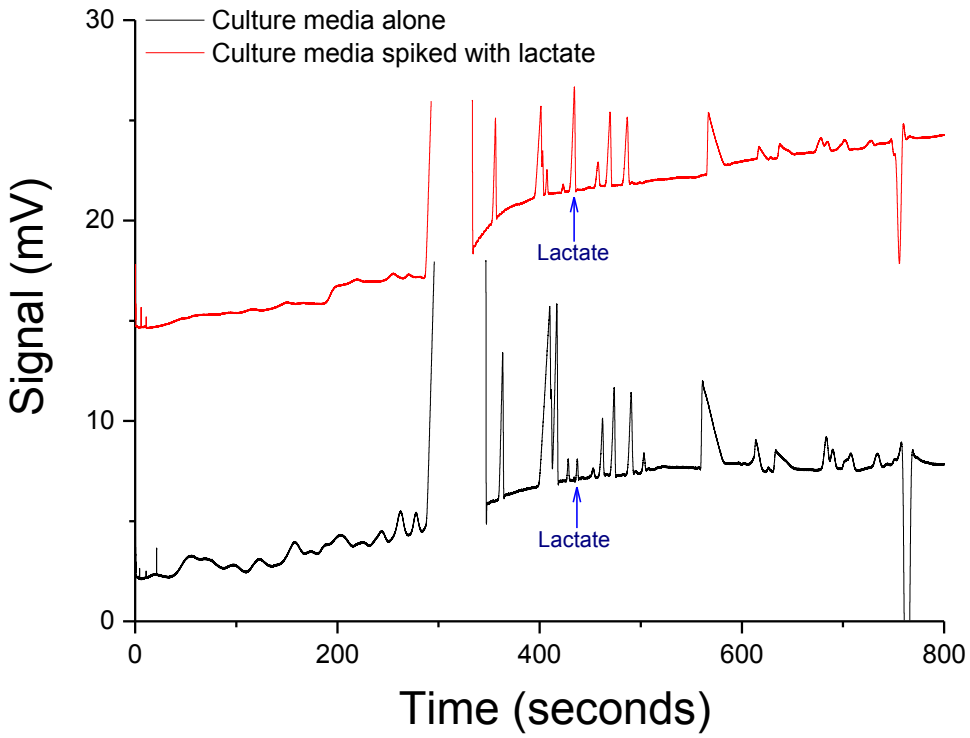


Figure 2-2 Electropherograms of cell culture media alone (black) and of the same media spiked with 0.125 mM lactate (red), Conditions: 85 cm x 50 µm I.D. fused silica capillary coated with HDMB/PSS/HDMB; BGE: 25 mM Tris/35 mM CHES, pH 8.85 with 0.02% PEI; +30 kV applied to outlet vial while interface was grounded. Signal was obtained using a TraceDEC conductivity detector C^4D positioned 10 cm from the outlet.

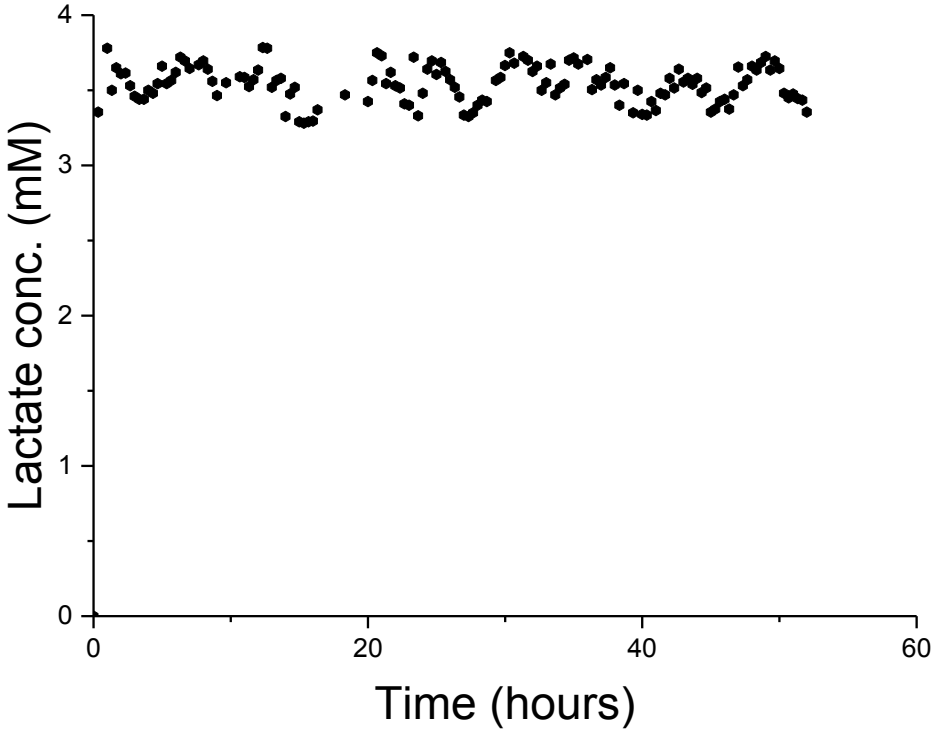


Figure 2-3 Monitoring of a standard solution containing 3.5 mM lactate over time. Conditions as described in Figure 2-2.

Displacement pumps provide suitable, pulsation-free alternative to introduce approximately equal amounts of sample each run as described by Tahkoiemi *et al.* for online bioprocess monitoring as mentioned above[23,29]. Displacement pumps, however, require a larger amount of sample (44 μL) for each run, more than five times the volume that can be achieved using peristaltic pumps. As the small sample volume is a crucial for the use of the proposed system for bioprocess monitoring, a correction for oscillations the *due to the use of a peristaltic pump* was made using chloride as internal standard to correct the lactate peak area. The level of chloride in the cell culture media (241 mM) is not expected to vary significantly over time despite the small but continuous flux of chloride into and out of cells to maintain ion homeostasis. The chloride peak area was found to vary by ± 5% over 225 runs over 3 days, indicating only minimal fluctuations in chloride over time.

2.3.2 Sampling interface

To chemically monitor the composition of the media in culture by CE, a method for cell-free sampling is required to avoid clogging of the capillaries and other fluidic connections. From the variety of flasks available, a standard flask with a surface area of 75 cm^2 was selected for seeding 1,000,000 cells using 20 mL media. A CO_2 laser engraver was used to engrave a hole in the culturing flask to serve as an access hole for the sampling interface, and this hole was sealed with sticky tape immediately after ablation to minimize the risk of contamination.

Viable HEK293 cells are, like most mammalian cell lines, adherent, which means they will attach to the bottom of the culturing flask. As a result, the media above the cells can be considered as cell-free, and is suitable for direct injection into the SI-CE system. To selectively sample from this cell-free layer of media, a sampling interface was developed from a piece of Teflon tubing. Using the CO_2 laser engraver, a 500 μm hole was ablated in a

1.588 mm O.D., 0.508 mm I.D. tube, spaced 500 μm away from the end of the tube Figure 2-4.

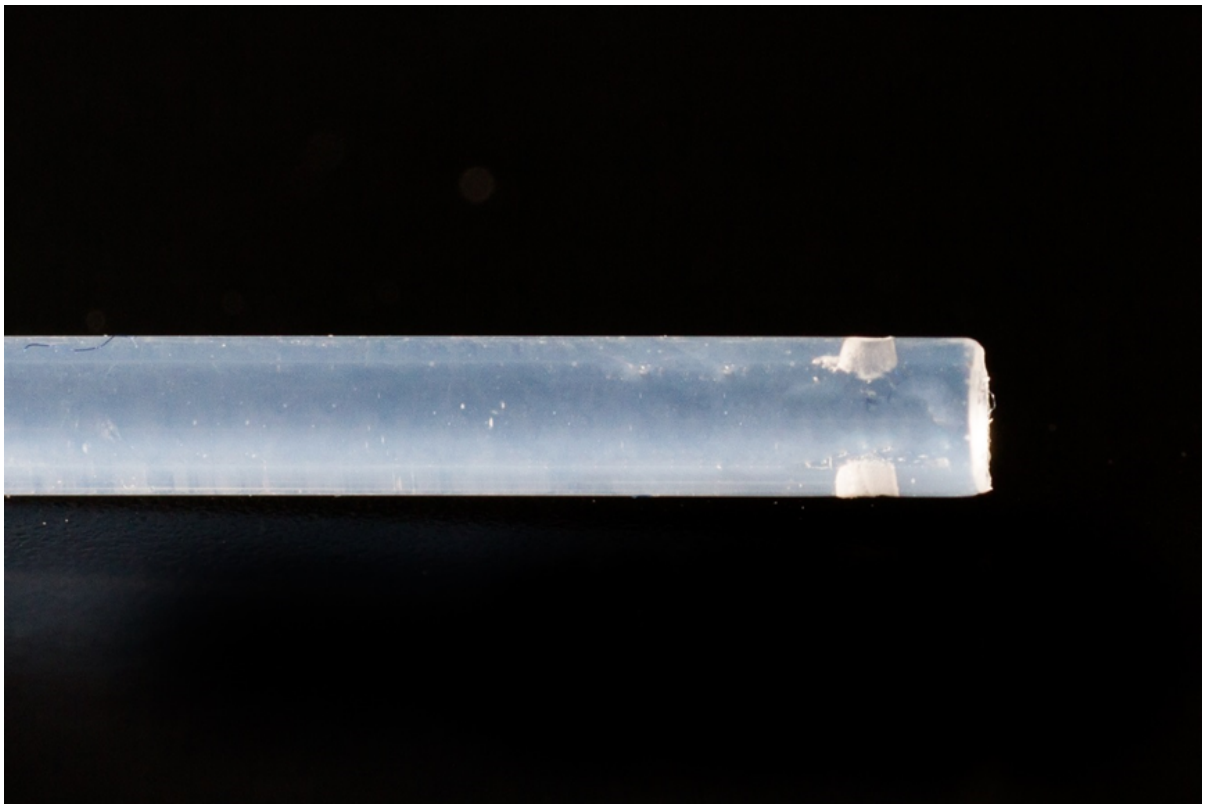


Figure 2-4 Photograph of the sampling interface consisting of a laser machined 500 μm hole, 500 μm from the edge of a piece of Teflon tubing (1.588 mm O.D., 0.508 mm I.D.).

This distance was selected to be sufficiently high above the cells to prevent any cells from entering the analytical system, and sufficiently low to ensure that media but not air was sampled for the duration of the experiment. This sampling tube was sterilized carefully with 70% ethanol before its insertion into the culturing flask.

For assembly, the tape isolating the hole in the cell culture bottle was removed from the culturing flask in a sterile biohazard cabinet and the tubing, sterilized with 70% ethanol, was fed through the hole and pressed firmly on the bottom to ensure a liquid seal. This seal is important to restrict the fluid intake to the sampling hole, preventing the cells from entering the sampling tube through the bottom of the tube (Figure 2-5). The sampling tube was then sealed in place with sterile aquarium grade silicon, and connected to the peristaltic pump.

Figure 2-5 Photograph of the Sampling tubing inserted through a 510 µm hole laser machined in a 75 cm^2 culture flask. The sampling tubing touches the bottom of the flask.

2.3.3 Monitoring of lactate production in HEK293 cells

Cells were loaded into the prepared culturing flask with sampling interface and left to adhere overnight before being washed with PBS twice before 20 mL Leibowitz L15 medium was placed in the flask. The adhesion of the cells to the bottom of the flask is crucial for sampling cell-free media. The flask was then immediately transferred to the humidified, 37 °C cell culture incubator and connected to the sampling and measurement set up. Here, a Y-connector was used to dilute the sampled cell culture media 1:1 with Milli-Q water before injection in the SI-CE system. This reduced protein content and served to halved the required sampling volume. Based on the process times reported in Table 2-1, a new sample was analysed every 20 min, resulting in a total of 228 electropherograms over 76 h. Figure 2-6 shows ten selected electropherograms from 20 to 200 min after the start of the sampling. For each analysis, only 8.73 μL of culture media was consumed, resulting in a total sample volume of 1.99 mL of media over three days – this is less than 10% of the total media present in the culture flask.

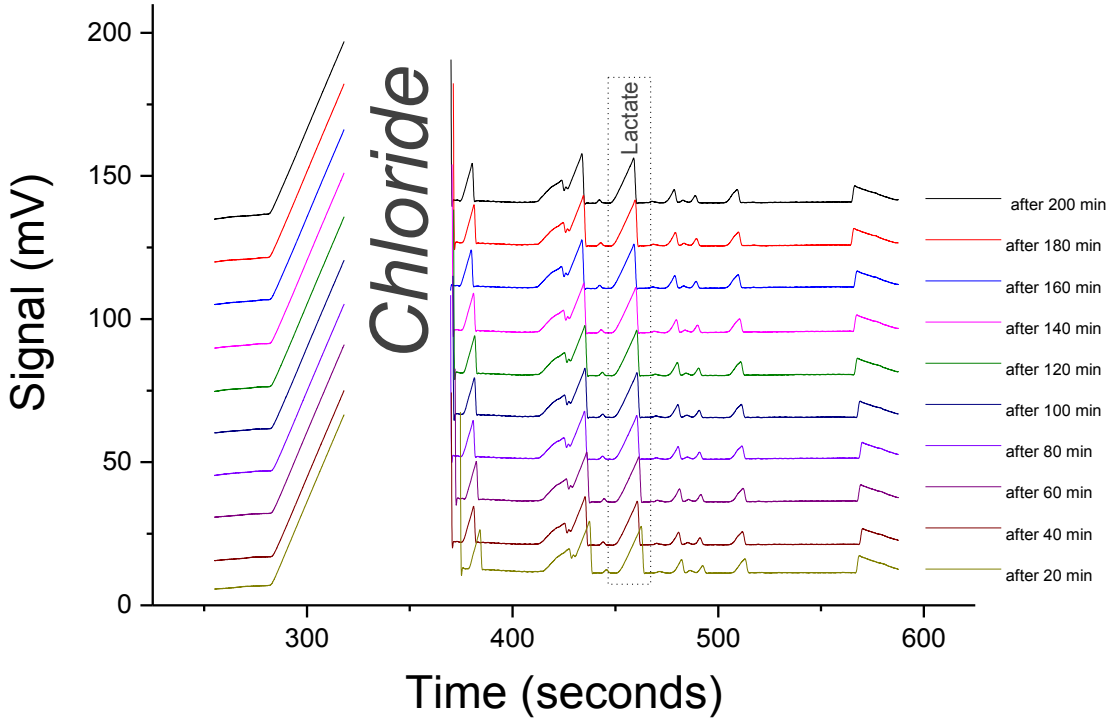


Figure 2-6 Monitoring lactate production of HEK293 cells. The image shows 10 consecutive electropherograms recorded between 20 and 200 minutes from the start of the experiment, conditions as in Figure 2-2.

While the gradual decrease in the amount of media in each run is small, there is still the possibility that this volume change will induce changes in concentration and contribute as a major error in the measurements. This was minimised by using chloride as internal standard to lower any possible variations in lactate level readings. However, both the amount of sample used for analysis and the sampling time intervals can be controlled according to the practical application and the volume of cell culture.

Figure 2-7 represents the change in lactate concentrations over the cell culture. During the first 30 h of culture, the lactate concentration in the culture media remained unchanged before increasing steadily at a rate of 0.03 mM lactate per hour. This likely represents the lactate production by HEK293 cells under mostly anaerobic conditions.

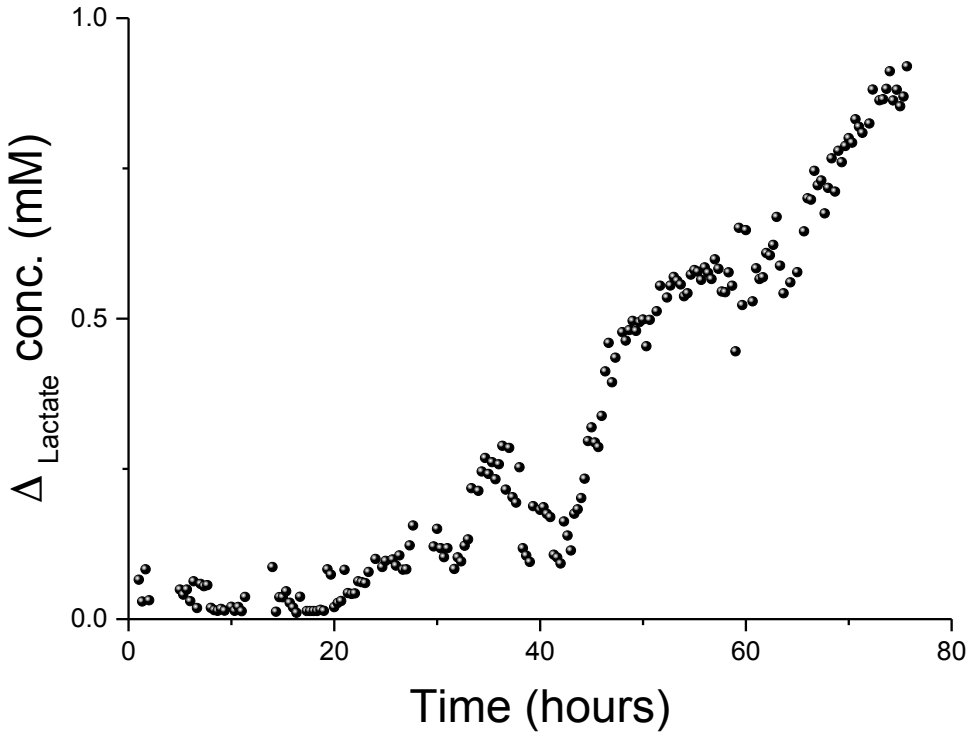


Figure 2-7 Lactate production of HEK293 cells over 76 hours, Peak areas corrected for changes in injection volume using Cl^- as internal standard. $\Delta_{lactate}$ = lactate (measured) - lactate (basal).

2.3.4 Monitoring of lactate production in mouse fibroblast cells

One of the main advantages of the presented system is the ability to show variations in lactate concentration over short time intervals. This feature is of importance to compare any change in the lactate production profiles between cells with different mitochondrial activity. Here, we measured lactate production in fibroblasts cell culture of a wild type (WT) mouse (control) and compared it with the lactate production profile from fibroblasts taken from another mouse with abnormal mitochondrial function, a mitochondrial gene ND4 knockout (ND4 is a subunit of mitochondrial respiratory chain complex 1). It was expected to observe higher lactate production rate in cells cultured from the mice with the abnormal mitochondrial function compared to the WT.

After leaving cells to adhere on the culture flask bottom surface, cells were washed with PBS and cultured with Leibowitz L15 medium before the transfer to the incubator containing the analytical system. Samples were taken and analysed every 20 min for 3 days. Figure 2-8 shows the change in lactate concentrations from each cell line.

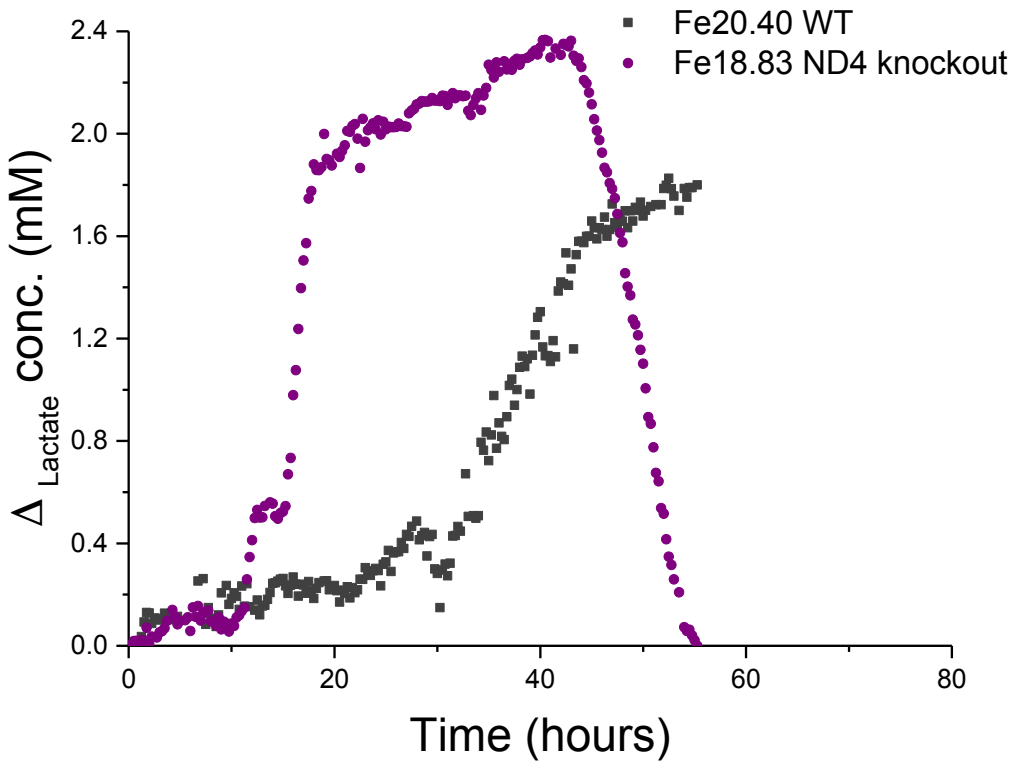


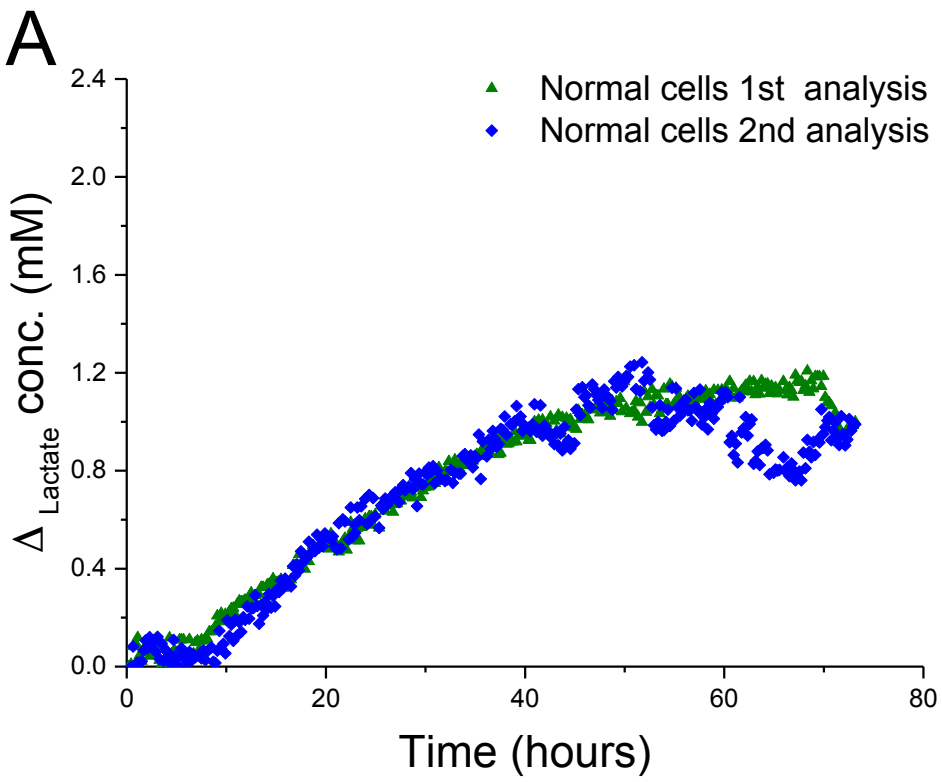
Figure 2-8 Lactate production of WT (Fe20.40) and ND4 knockout (Fe18.83) cell lines over 56 hours, peak areas corrected for changes in injection volume using Cl^- as internal standard. $\Delta_{lactate}$ = lactate (measured) - lactate (basal).

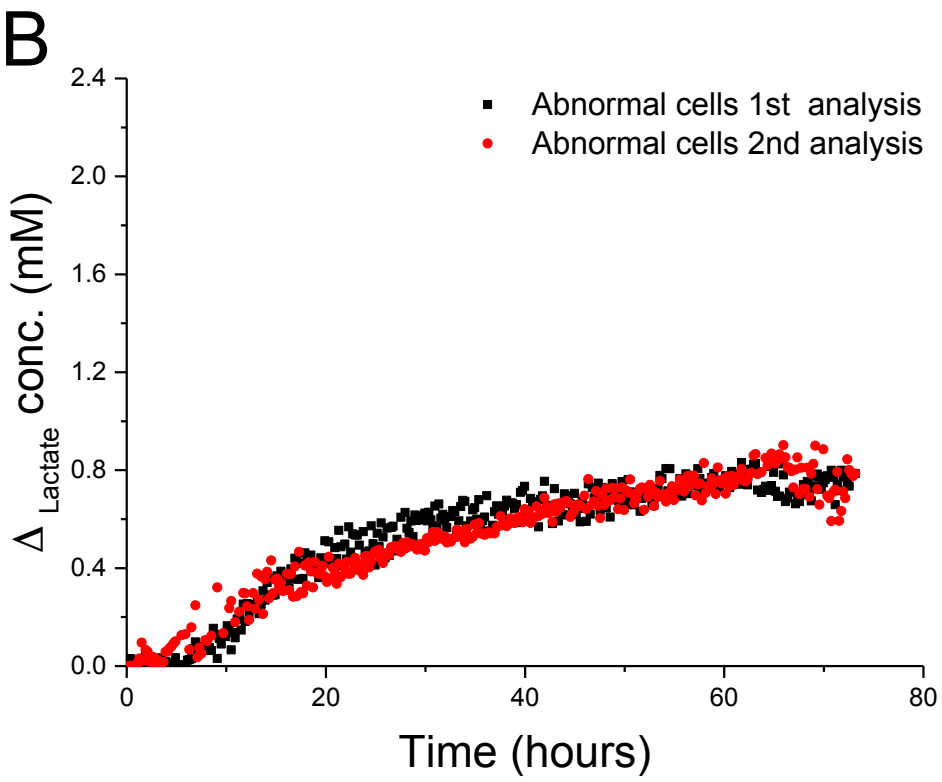
As expected, the lactate production in the ND4 knockout cell line occurred at a higher rate than in the WT. In the first 10 h of culture, lactate was produced in a similar rate in both cell lines. An elevated high rate of lactate production was noticed in the time period between 10 – 20 h in the ND4 knockout cell line (216 μM/h) compared to the WT (8 μM/h). Between 20 – 43 h a steep increase in lactate production was observed for the WT (55 μM/h) whereas over this time interval the lactate production in the ND4 knockout had slowed down to 18 μM/h. Then a sudden decrease in lactate production was recorded in the MD4 knockout from 43 h to the end of culture.

To repeat this experiment, new cell lines were obtained from other mice, again one WT and one ND4 knockout cell line. This time, the monitoring study was carried out twice, interrupted shortly for between runs to wash and provide fresh media to the cells attached on the culture flask, to assess the reproducibility of lactate profile for each cell line. Figure 2-9A shows the lactate production by the WT cell line for the two runs, demonstrating the lactate production rate was statistically similar ($p = 0.104$) between the two runs. The ND4 knockout cell line also showed a high degree of similarity in lactate production between repetitions ($p = 0.905$) (Figure 2-9B). The similarity of the profiles provides between the two repeats provides confidence in the presented approach.

Surprisingly, comparing lactate profiles between the WT and ND4 knockout cell lines contradicts the trend observed in the earlier experiment (Figure 2-9C). In both repeats, the lactate production rate in the ND4 knockout cell line was (10 μM/h), compared with 16 μM/h in the WT (values reported as average of both repetitions).

At this point, the data are non-conclusive and the experiment was repeated. Unfortunately, an infection compromised this experiment and killed the cell lines. No new cell lines could be obtained, which led to the termination of this study.


A
Normal cells 1st analysis
Normal cells 2nd analysis
Δ Lactate conc. (mM)
2.4
2.0
1.6
1.2
0.8
0.4
0.0
0
20
40
60
80
Time (hours)


B
Abnormal cells 1st analysis
Abnormal cells 2nd analysis
Δ Lactate conc. (mM)
2.4
2.0
1.6
1.2
0.8
0.4
0.0
0
20
40
60
80
Time (hours)

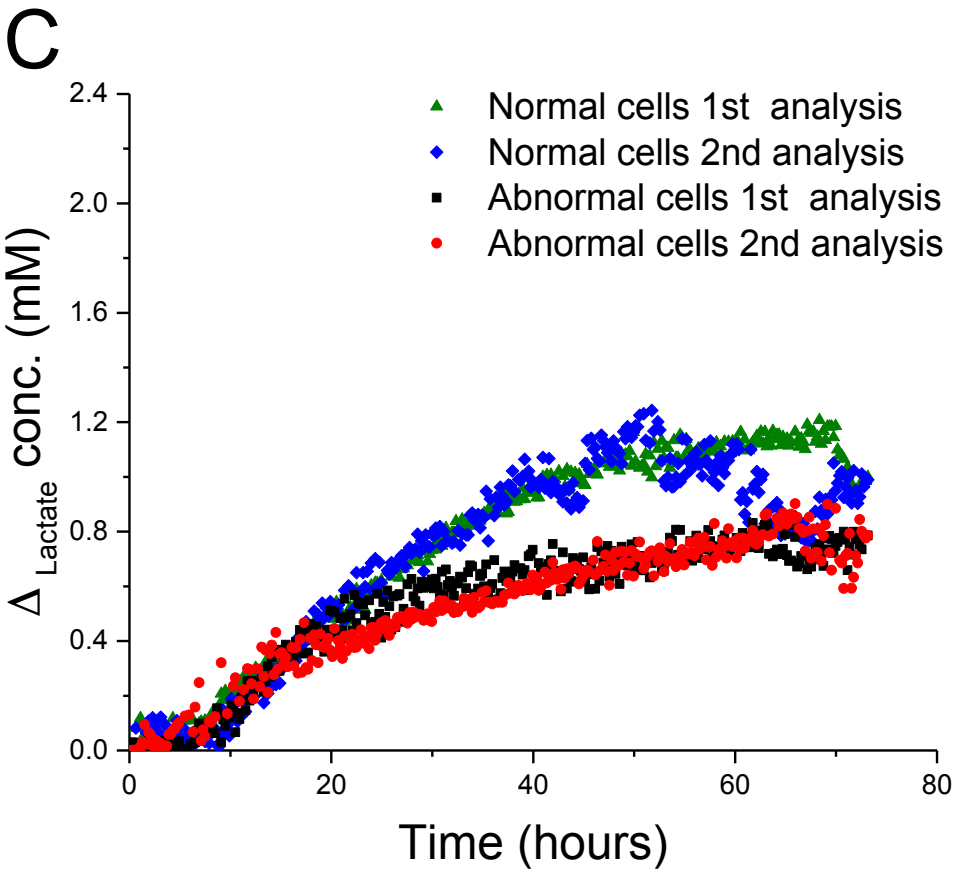


Figure 2-9 Lactate production of WT (Fe20.40) and ND4 knockout (Fe18.83) cell lines over 73 hours, A: Repeat experiments in the WT cell line, B: Repeat experiments in the ND4 knockout, C: Comparison of lactate production in WT and ND4 knockout cell lines. Peak areas corrected for changes in injection volume using Cl^- as internal standard. $\Delta_{lactate}$ = lactate (measured) - lactate (basal).

The observed time-dependent change in lactate production, as well as the rate of lactate production provides valuable insights into the cell culturing process, and would not have been observed using end-point measurements. The elucidation of the changes in lactate production, and hence cellular metabolism and energy levels over time will lead to a deeper understanding and improved control of bioprocesses.

2.4 Conclusion

An online, automated system for monitoring lactate in cell culture is presented, consuming less than 10% of media (1.99 mL), or only 8.73 μL of sample per run, over a three-day period (76 h). A flow-through sequential injection capillary electrophoresis system was connected to a sampling interface to inject cell-free media from an adherent cell culture into the analytical system and applied for near-time monitoring of the production of lactate in mammalian cell culture. The system is flexible and could be up- and out-scaled, with developments in separation chemistry allowing for the expansion of the analyte range and/or increase of the analysis frequency whilst small changes in hardware would enable monitoring of multiple cultures in parallel. The capability of online monitoring of bioprocesses introduces a new era in cell culture experiments, replacing end-point analytical assays with insights into temporal changes in the extracellular media, and hence in cell health. This will provide the data required for improved understanding of biological processes, for example in elucidating pharmacological mechanisms, as well as in optimizing and regulating biopharmaceutical production.

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Chapter 3

Capillary Electrophoresis for Automated On-line Monitoring of Suspension Cultures: Correlating Cell Density, Nutrients and Metabolites in near real-time[3]

3.1 Introduction

Bioprocess monitoring has gained importance over the past few years[1]. The production of chemicals or biopharmaceuticals using biological processes is naturally susceptible to variability because living cells consume substrates and produce metabolites and products in a dynamic way with variations in metabolic rate across short time intervals. The FDA recommends documentation of nutrient and metabolite time profiles in the process analytical technology (PAT) policy to ensure product quality[2]. Whilst bioreactors are typically equipped with hardware for monitoring a range of physicochemical variables including pH, temperature and dissolved oxygen[3], cell count and the analysis of metabolite levels are usually conducted off-line[4,5], making it difficult to control production in a quality-by-design manner. Recent advances in multivariate analysis in combination with spectroscopic techniques can provide more detailed chemical information, for example using UV[6] FTIR[7] or RAMAN spectroscopy[8,9]. Whilst advances in chemometry and the interpretation of spectra have greatly advanced, the resolution of complex analyte sets with similar functional groups remains challenging. Separation science provides many solutions for the analysis of complex samples, for example using high performance liquid chromatography (HPLC) offline[10] or on online[11]. Though mass spectrometry offers faster analysis, like other spectroscopic techniques

[3] Update from an article accepted in, **Alhusban, A. A.; Breadmore, M. C.; Gueven, N.; Guijt, R. M., Capillary electrophoresis for automated on-line monitoring of suspension cultures: correlating cell density, nutrients and metabolites in near real-time**, *Analytica Chimica Acta* **2015, ACA-15-2656, xxx-xxx.**

the ability to analyse complex samples, or to distinguish isomers with identical mass sequentially without extensive sample clean-up limits its applicability. Additionally, once an automatic sampling routine has been established, the analyte set can easily be expanded by changes to the separation chemistry and/or detector.

Capillary electrophoresis (CE) is an alternative high-resolution separation technique to HPLC with the potential to provide detailed chemical information faster and using smaller sample volumes[12]. In a recent example, using off-line derivatisation with a fluorescent label, Turkia *et al.* used capillary electrophoresis for monitoring the uptake of amino acids in beer brewing[13]. For automated, on-line monitoring, the same group constructed a CE system with flow-through sampling vial and cross filtration unit for online monitoring of the production of carboxylic acids by two different yeasts cultures. In their work, the yeast *kluyveromyces lactis* cultivation was monitored for 173 hours and 0.87 L of sample was used for 97 analyses (8.94 mL per analysis)[14].

Here, a robust and reliable electrophoretic separation method was developed for the analysis of the metabolic biomarkers glucose, glutamine, leucine/isoleucine and lactate from media and combined online with sampling and cell density measurements. The platform, schematically depicted in Figure 3-1 was used to monitor cell density and these four biomarkers in a culture of human T lymphocytes every 30 minutes over 4 days, using less than 41 µL of sample per assay. The highly flexible platform is automated, fast and reliable and as such expected to be able to provide new insights in the way chemical changes influence the production of biotechnology products, including biopharmaceuticals, and will enable the establishment of boundary conditions ensuring their quality.

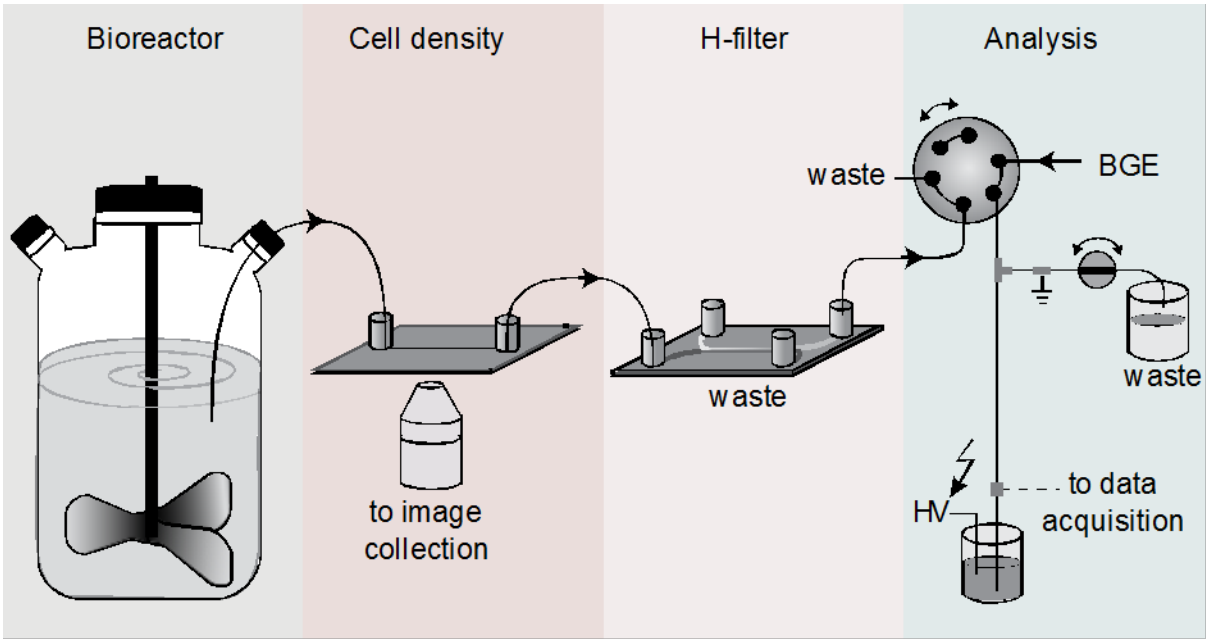


Figure 3-1 Platform used for near real-time monitoring of Jurkat cells, the cells were cultured under standard conditions in a bioreactor. Microfluidic devices were used to measure cell density and to derive a cell-and particulate free sample for analysis by SI-CE.

3.2 Materials and Methods

3.2.1 Chemicals

All reagents were analytical grade obtained from Sigma-Aldrich (Sydney, AUS) and were used as supplied unless stated otherwise. Milli-Q water (Millipore, Bedford, MA, USA) was used to prepare solutions. Standards containing 0.30 mM sodium fluorescein or 0.42 mM rhodamine-6G (BDH Chemical Ltd, Poole, UK) were prepared in milliQ water and stored at room temperature in a dark place. Fresh standards containing 10 mM lactate, 10 mM L-glutamine, 5 mM L-leucine, 5 mM L-Isoleucine or 40 mM D-glucose standard solution were prepared each week and stored at 8 ºC. A 2 M chloride standard was prepared monthly by dissolution of its sodium salt and stored at room temperature. The cationic polyelectrolytes poly(ethylenimine) (PEI) (ACROS organics, Geel, Belgium) at 0.075% (w/v) and hexadimethrine bromide (HDMB) at 0.005% (w/v) were added to BGE. A 90 cm PMMA capillary was coated with a polyelectrolyte coating prepared from (HDMB) and poly(sodium 4-styrene sulfonate homopolymer) (PSS) and (HDMB) to reverse the EOF. The BGE included a combination of 60 mM triethylamine (TEA) and 10 mM N-cyclohexyl-3-aminopropanesulfonic acid (CAPS).

3.2.2 Fabrication of H-filter

Templates were made using dry film resist with slight modifications[15]. A PMMA slide (80 × 60 × 1 mm), which were rinsed with methanol, dried using compressed air were laminated sequentially with three 0.1 mm EP SUEX TDFS sheets (Sudbury, MA, USA) at 60 °C and cooled down with compressed air. The protective film was removed before applying the next layer. After removal of the final protective coversheet of the top film, a transparency mask, made by laser printing on an overhead transparency, was positioned on top and the substrate was exposed for 21 minutes to the output of a UV LED array (OTHL-0480-UV, Opto

Technology, Wheeling, IL, USA)[16] and then baked at 110 °C according to the SUEX processing guidelines. Substrates were developed in propylene glycol monomethyl ether acetate (PGMEA), followed by a wash with isopropanol and then dried with compressed air. The microfluidic H-filter devices were created by casting polydimethylsiloxane (PDMS) using soft lithography with the PDMS cured for 1 hour in an oven at 70 °C. Access holes were made at the end of each channel using a hole puncher and the device was irreversibly sealed by plasma bonding with a glass microscope slide (75 × 51 × 1 mm). A digital fluorescence microscope (AM4113/AD4113, Dino-lite premier digital microscope, Taiwan) was used for testing the fabricated device.

3.2.3 Cell culture

The human T lymphocyte cell line (Jurkat ATCC® TIB-152) were routinely cultured in cell culture flasks at 37 ºC in a cell culture incubator without CO_2, in RPMI-1640 Medium (25 mM HEPES, L-glutamine without $NaHCO_2$; 5% fetal calf serum; FCS, VWR, Murarrie, Australia). For measurement of glucose, glutamine, leucine/isoleucine and lactate, 45 million cells were centrifuged for 5 minutes at 200 g at room temperature before the pellet was re-suspended gently in 30 mL culture medium and transferred into a 250 mL double sidearm Celstir® spinner flask (Thomas Scientific, Swedesboro, NJ, U.S.A) to form a total volume of 175 mL, equivalent to 7.5 x 10^5 cells.mL^{-1}. The pre-assembled culture device including the sampling tubing (inserted through a hole in the coverlid), bioreactor, coverlids, peristaltic pump tubing and the H-filter device, was autoclaved as one unit prior to its use.

3.2.4 Cell density measurement

Cell counting microscopy chambers (1µ-slide$VI^{0.1}$, Ibidi GmbH, Martinsried, Germany) were treated with 5 mL 10% HCl (v/v) several times at room temperature and left overnight to remove the Poly-L-Lysine coating to prevent cell adhesion between runs. They were

subsequently washed several times with MilliQ water and rinsed with 70% ethanol before use. A prepared microscopy chamber was fixed onto the stage of a phase contrast microscope (Eclipse TS100, Nikon, Japan) equipped with a digital camera (AM7023B Dino-Eye, New Taipei City, 241 Taiwan) to capture images for cell counting. Images of cells flowing from the bioreactor through the counting microscopy chamber were automatically captured using software written with LabView software v8.1 (National Instruments) every 30 minutes at 40 x magnification and coincided with injection into the capillary electrophoresis instrument. The resulting images were analysed based on % surface area covered by cells or multicellular aggregates. The image analysis procedure comprised cropping the image to remove unrelated area, converting the image to black and white, adjusting the black colour intensity and finally determining the % surface area covered by cells or cell aggregates in the adjusted image (Figure 3-2) and was conducted using the freeware package Image J.

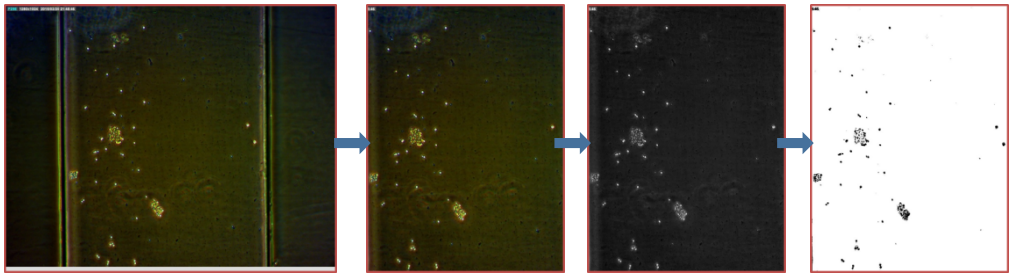


Figure 3-2 Analysis for automated assessment of cell density using ImageJ software;

The following steps were followed in image analysis

Open image → Image/Crop → Image/Type/Select 8 bit (to convert the image into BW image) Image/Adjust/Threshold: "black background" top bar at 85, lower bar at 255, default, back and B&W then Apply Analyze/Analyze particles, Size 10-Infinity, Circularity 0-1, Show Masks, select all except "record stats" → get the % Surface area→ OK

3.2.5 Instrument Design and Operation

The SI-CE instrumentation is based on previous designs used in our laboratory[17,18]. It is composed of two peristaltic pumps (PeriWaves, CorSolutions, Ithaca, NY, USA) for both sample and BGE delivery. The H-filter was positioned between the sample pump and injection valve to prevent cells from clogging the valve. A three way solenoid valve (360T041SHH, NResearch, West Caldwell, NJ, USA) was used to direct the extracted sample solution into the injector or waste. A high-pressure two-position six way switching valve (MXP9900-000, Rheodyne, Oak Harbor, WA, U.S.A) was used to control the delivery of the BGE or sample to the capillary interface. A PEEK T-piece-connector (P-727, Upchurch Scientific, OakHarbor, WA, U.S.A.) was used to interface the separation capillary with the flow injection system. A PMMA capillary (28 μm I.D.; Paradigm Optics, Vancouver, WA, U.S.A) served as separation capillary and its inlet was fixed in a position to minimize carry over. The outlet end was immersed in a 25 mL glass vial filled with BGE. A stainless steel syringe needle was used as hollow electrode and connected the T-interface with the 1.5875 mm O.D., 0.508 mm I.D. Teflon tubing leading to the waste. A two way solenoid valve (HP225K021, NResearch, West Caldwell, NJ, USA) was connected with this tubing to control the flow between the separation capillary and waste (Figure 3-3). Capacitively coupled contactless conductivity detection, C^4D, was used because of its demonstrated ability to detect optically transparent analytes like lactate for monitoring exhaled breath[19] or adhesion cultures[18].

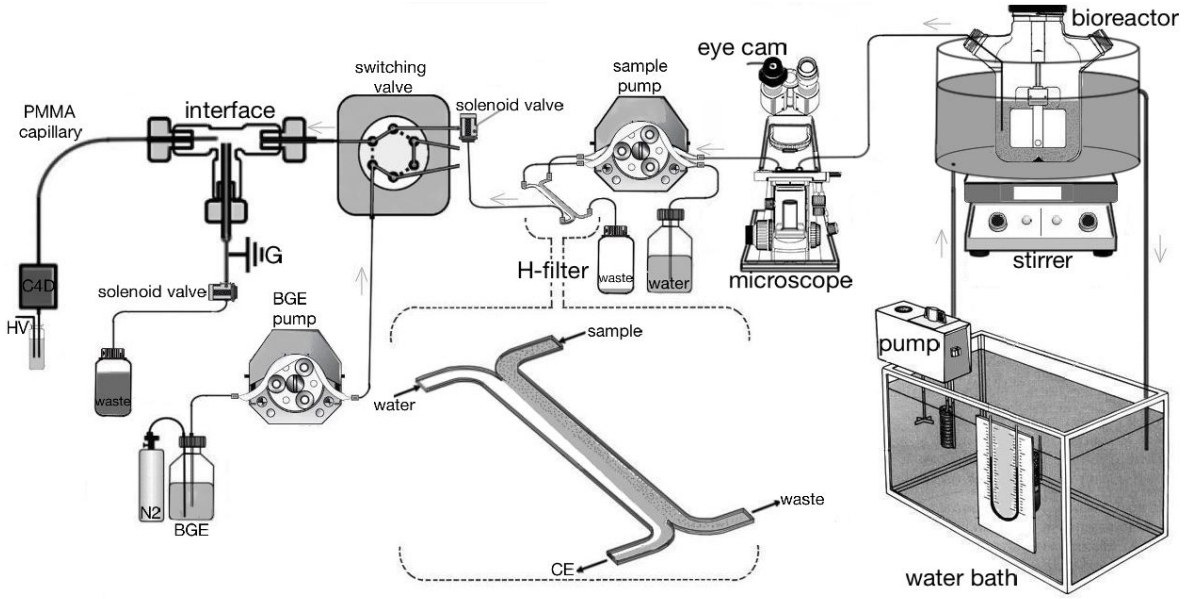


Figure 3-3 Schematic diagram of the experimental setup placed inside a laminar flow cabinet. The sample is withdrawn from the 37 °C bioreactor. Cell density in the sample is measured using automated image analysis of images captured by a digital microscope before the sample enters the H-filter. The cell-free H-filter effluent is analysed by SI-CE using C^4D for detection of substrates and metabolites.

The operational parameters for the C^4D were optimized as follow: frequency, 2x high; voltage, 18 Db; gain, 200%; off set, 000. The anode (+) electrode of a reversed polarity high-voltage power supply (4300 Emco, Sydney, NSW, Australia) was positioned in the outlet BGE vial. A NI USB-6212 data acquisition interface board (National instruments, Austin, TX, U.S.A.) was controlled using LabView v8.1 (National Instruments) to control the pumps, switching valve, solenoid valves and power supply. The operation of the SI-CE setup was as follows: both the T-interface and capillary were filled and flushed with BGE for equilibration and cleaning purposes. The three way solenoid valve (solenoid connected to the H-filter) was opened to flush the contents of the H-filter to waste and closed to deliver the cell-free media sample into the switching valve. The switching valve was switched to direct sample to the T-interface and the two way solenoid valve was briefly closed to hydrodynamically inject the sample into the separation capillary. The switching valve was switched back to direct BGE towards the T-interface, which was cleaned with BGE at a high flow rate. A high voltage (+30 kV) was applied for electrophoretic separation of the sample. The sequence of events was carefully optimized and the optimized conditions are detailed in Table 3-1. All separations were performed at ambient temperature (20 °C).

Table 3-1 CE system sequence of events, the platform was operated by Labview.

	Operation	Position switching valve	Position 3-way solenoid	Position 2-way solenoid	Flow rate ($\mu L.min^{-1}$)	Time (s)	BGE used (µL)	Sample used (µL)
1	*Flushing of capillary with BGE*	1	Open	Close	50	180	150	0
2	*Equilibrate*	1	Open	Open	0	1	0	0
3	*Activation H-filter*	2	Open	Open	8	210	0	28
4	*Filling interface with sample*	2	Close	Open	8	85	0	11.33
5	*Hydrodynamic injection*	2	Close	Close	15	5	0	1.25
6	*Filling interface with BGE*	1	Open	Open	250	15	62.5	0
7	*Separation*	1	Open	Open	75	1304	1630	0

A double sidearm Celstir® bioreactor was used for culturing the T-cells. It was placed in a plastic container above a magnetic stirrer (Townson and Mercer, Manchester, UK) and connected to a water bath (Techne, Duxford, Cambridge, UK). A pump was used to circulate water at 37 °C continuously through the plastic container to thermostat the bioreactor. A 1.59 mm diameter hole was drilled into the coverlid of the flask sidearm to insert 1.5875 mm O.D. Teflon tubing for sampling. This tubing was connected to the inlet of a counting chamber placed on a microscope with digital camera (AM7023B Dino-Eye, New Taipei City, 241 Taiwan) to capture images for cell counting. The outlet of the counting chamber was connected to a peristaltic pump and the setup was placed in a laminar flow cabinet (ESCO, Singapore) to minimize the risk of contamination.

3.2.6 Electrophoretic Conditions

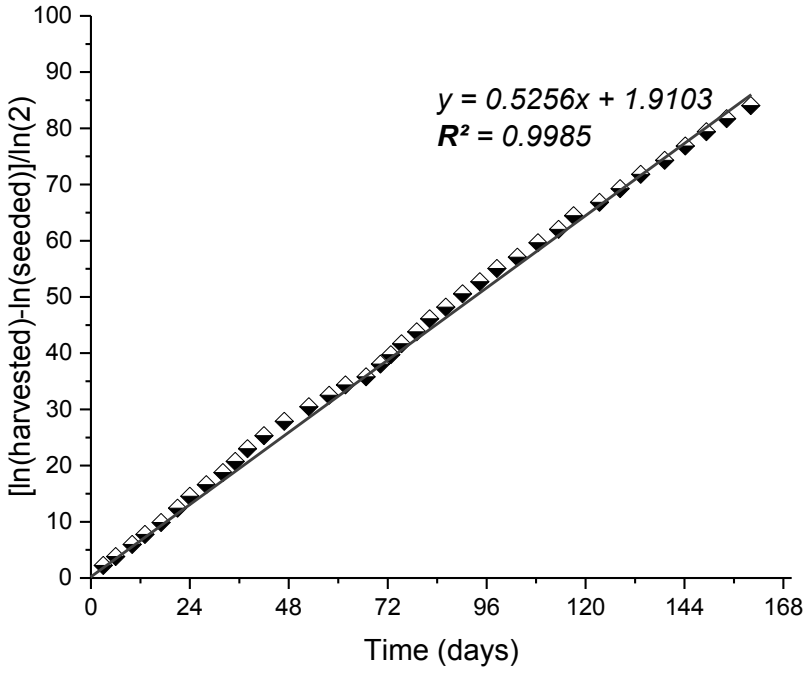
PMMA capillaries with 90 cm length (L_D = 80 cm) were used. Capillaries were flushed with ethanol for 1 hour followed by 2.5 mM NaOH at 0.5 $\mu L.min^{-1}$ for 20 min, then Milli-Q water at the same flow rate for 5 min. After that, the capillaries were flushed with 1% aqueous solution of HDMB for 10 min at 0.5 $\mu L.min^{-1}$, Milli-Q water at 0.5 $\mu L.min^{-1}$ for 5 min, 1% PSS for 10 min at 0.5 $\mu L.min^{-1}$, Milli-Q water at 0.5 $\mu L.min^{-1}$ for 5 min, 1% aqueous solution of HDMB for 10 min at 0.5 $\mu L.min^{-1}$. After this procedure, the capillaries were left for at least three days to strengthen the polyelectrolyte coating, and the coated capillary was flushed with BGE for 30 min prior to use. The BGE consisted of 60 mM triethyamine (TEA) and 10 mM cyclohexyl-2-aminoethanesulfonic acid (CAPS) at pH 12.40. 0.005% HDMB (w/v) and 0.075% (w/v) PEI were added to stabilize the polyelectrolyte coating and to adjust selectivity. Electrophoretic separations were conducted at -30 kV. The BGE bottle was connected to a nitrogen gas line to prevent alteration of the pH from CO_2 absorption.

3.3 Results

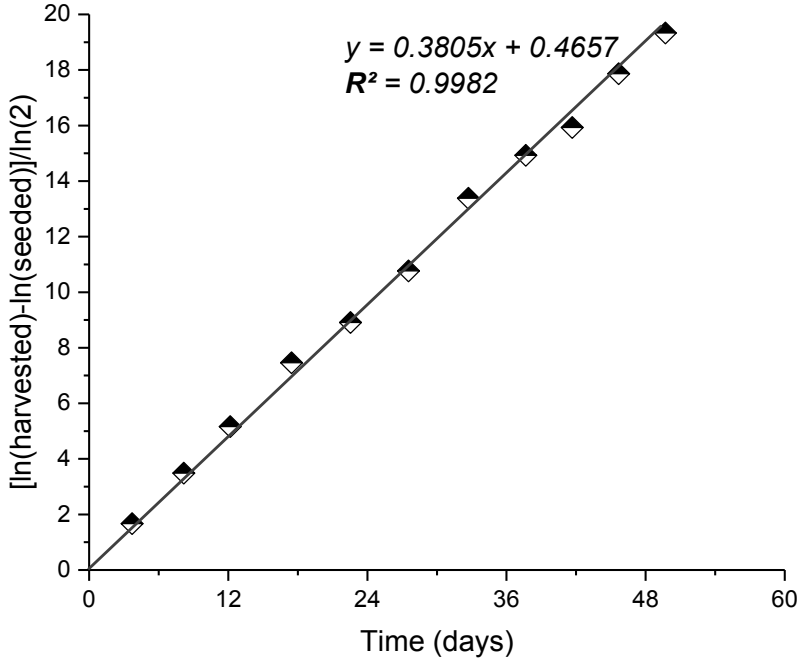
3.3.1 Cell culture optimization

Selecting the appropriate conditions required for cell growth is crucial. The recommended media for culturing Jurkat cells is RPMI-1640 medium with 10% FCS at 37 °C in 5% CO_2 incubator. High growth rate of cells in preliminary studies required lowering the amount of FCS to 5% and decreasing the starting density to 0.5 x 10^5 cell.mL^{-1} to enable passaging every 4 days. Monitoring cell growth status and viability for several weeks, different media were tested to allow the growth of cells in CO_2 free environment which is required for the electrophoresis system. RPMI-1640 with HEPES, 5%FCS and starting density of 0.75 x 10^5 cell.mL^{-1} were the optimized conditions for healthy growth. The most challenging step was to optimize cell growth and viability in the bioreactor flask that was surrounded by 37 °C flowing water. Here, the position of the bioreactor, the spinning rate, amount and content of media, rate of water flow to the plastic container that contained the flask, temperature control and other parameters were also optimized to make sure that cells were growing at a relatively constant rate without variation in viability. The growth state was estimated in term of cumulative population doublings in the three environments; CO_2 incubator (Figure 3-4A), CO_2 free incubator combined with bicarbonate free media (Figure 3-4B) and a water bath combined with bicarbonate-free media (Figure 3-4C), in which cell viability was determined by trypan blue assay (Figure 3-4D). The used media was specifically formulated to be buffered without CO_2, a method commonly used in large-scale bioreactors, for other applications were CO_2 might be essential for cell growth, the bioreactor could be connected to a CO_2 cylinder which will enable the flow of constant CO_2 to keep carbonate level in the media required for culture growth.

A


y = 0.5256x + 1.9103
R² = 0.9985
[ln(harvested)-ln(seeded)]/ln(2)
Time (days)
0
10
20
30
40
50
60
70
80
90
100
0
24
48
72
96
120
144
168

B


y = 0.3805x + 0.4657
R² = 0.9982
[ln(harvested)-ln(seeded)]/ln(2)
Time (days)
0
2
4
6
8
10
12
14
16
18
20
0
12
24
36
48
60

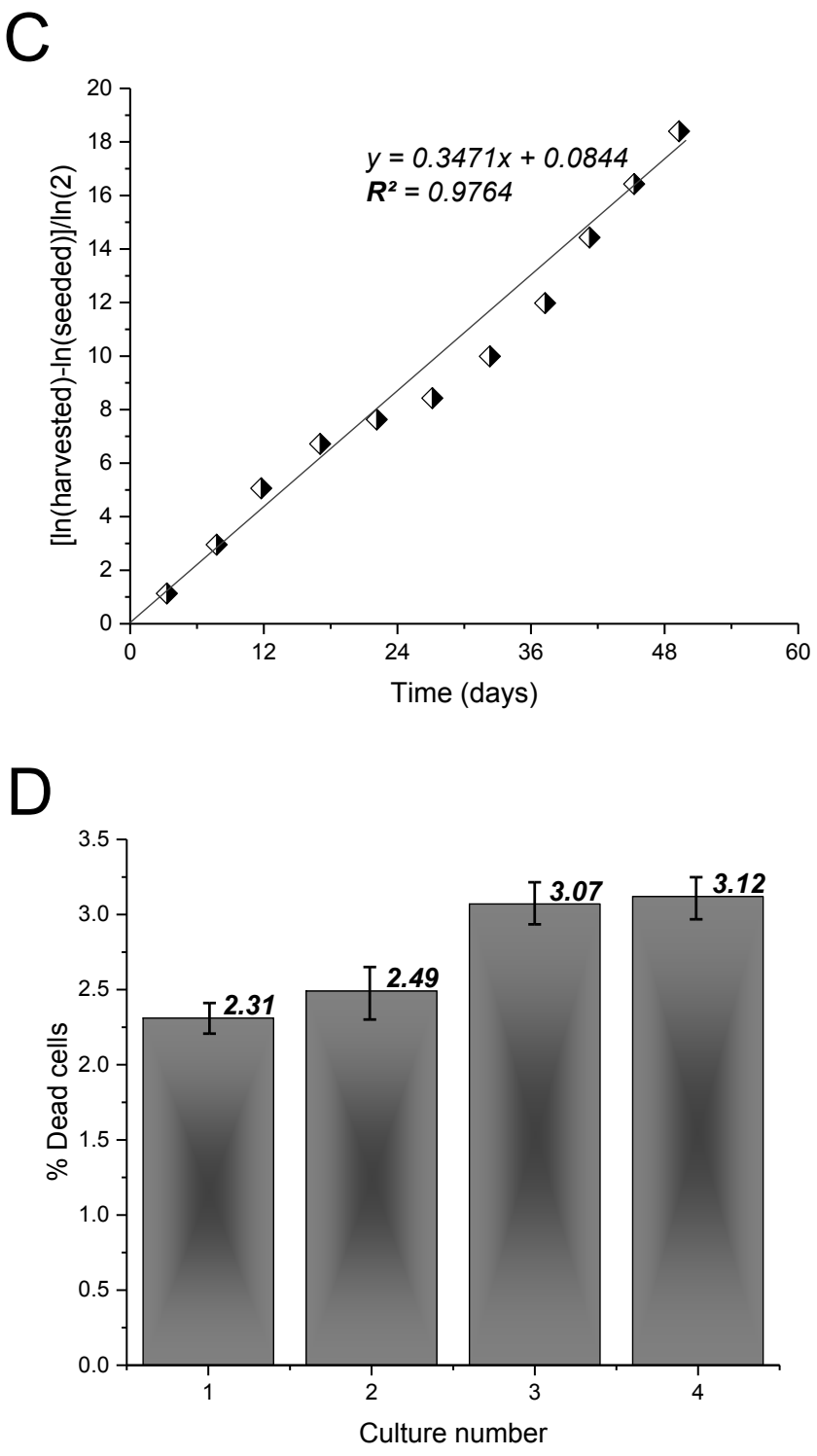


Figure 3-4 Cumulative population doublings of Jurkat cells in A: CO_2 incubator, B; CO_2 free incubator, C: water bath at 37 °C and D: % dead cells determined using Trypan blue assay.

3.3.2 Automated cell counting

Cell density is a key parameter to understand changes in nutrients and metabolites and correlate these to changes of cellular metabolism. Traditional cell counting using a haemocytometer is tedious and time-consuming, lacks statistical robustness and the validity of results is often compromised by counting errors and variations between investigators[20]. Flow cytometry provides an alternative to count cells more quickly and with superior reproducibility compared to manual counting. However, expensive equipment, specialized technical expertise regarding the repeated calibration and instrument software are essential to generate reliable data[21]. Digital imaging of cells in a flow chamber is gaining popularity as an alternative for cell counting as it minimises human errors, time and therefore cost[22].

In this study, a microfluidic counting chamber and digital microscope were used for automated counting of Jurkat cells. A prepared microscopy chamber was fixed onto the stage of a phase contrast microscope equipped with a digital camera to capture images for cell counting every 30 minutes. The resulting images were analysed based on % surface area covered by cells (Figure 3-5).

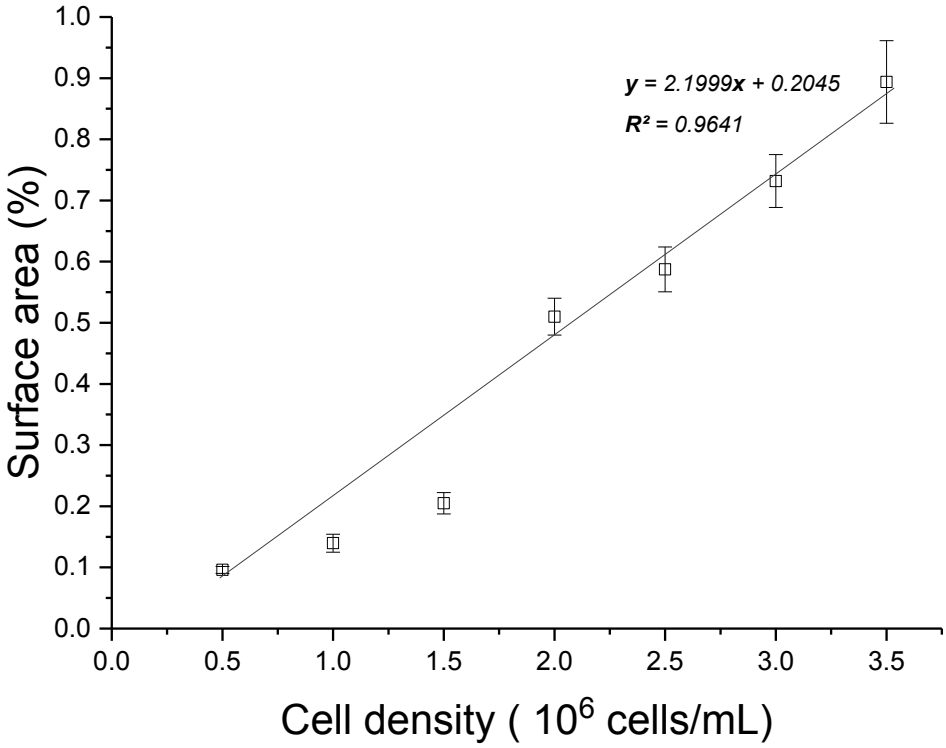


Figure 3-5 Image analysis protocol, expressed as % surface area covered by cells, calibration and validation with measurements made manually using a haemocytometer for cell density determination.

This approach cannot discriminate between live and dead cells, but with the % of dead cells determined to range from 2.3-3.1% (Figure 3-4D), this was not considered to be a concern. Seven cells densities ranging from 0.5-3.5 million cells.mL^{-1} were measured in triplicate and used to validate the automated counting procedure. Good linearity (r^2= 0.9641) and precision (RSD range 3.16-8.41) were obtained (Figure 3-5).

Over a period of 78 hours, the % surface area covered determined by image processing correlated well with the data obtained by manual counting of samples taken from the same culture using a haemocytometer. Beyond 78 hours, cell clumping, especially when the density exceeded three million cells.mL^{-1}, complicated the manual counting and the results from the manual counting indicated that the cells had reached a plateau phase of growth. The % coverage data obtained through image processing, however, suggested that growth continued linearly (Figure 3-6). The reported difficulties with counting the clumped cells by haemocytometry suggest that the digital image processing method might be more accurate and reliable at these high cell densities. For other commercial cell cultures such as prokaryotic cells, which might be run at high densities as high as 100 cells.mL^{-1}, cell clumping could be overcome by diluting the media withdrawn from the culture with 2x PBS solution. This will decrease the aggregation between individual cells and will keep cells amount passing the counting device within the calibrated range. Though, the phase contrast used has given an indication to distinguish between living and dead cells. For accurate life-dead cell differentiation, the use of fluorescence microscopy combined with commercially available life-dead stains such as kits from Promega, in which life cells are green and dead cells are red, would be a suggested solution. Besides, a more quantitative method would be to measure accumulated fluorescence over a certain amount of time or volume by attaching the system to a flow cytometer that can quantify total fluorescence in red and green channels in a defined volume. Here, the % viability would be represented by the ratio of red to green.

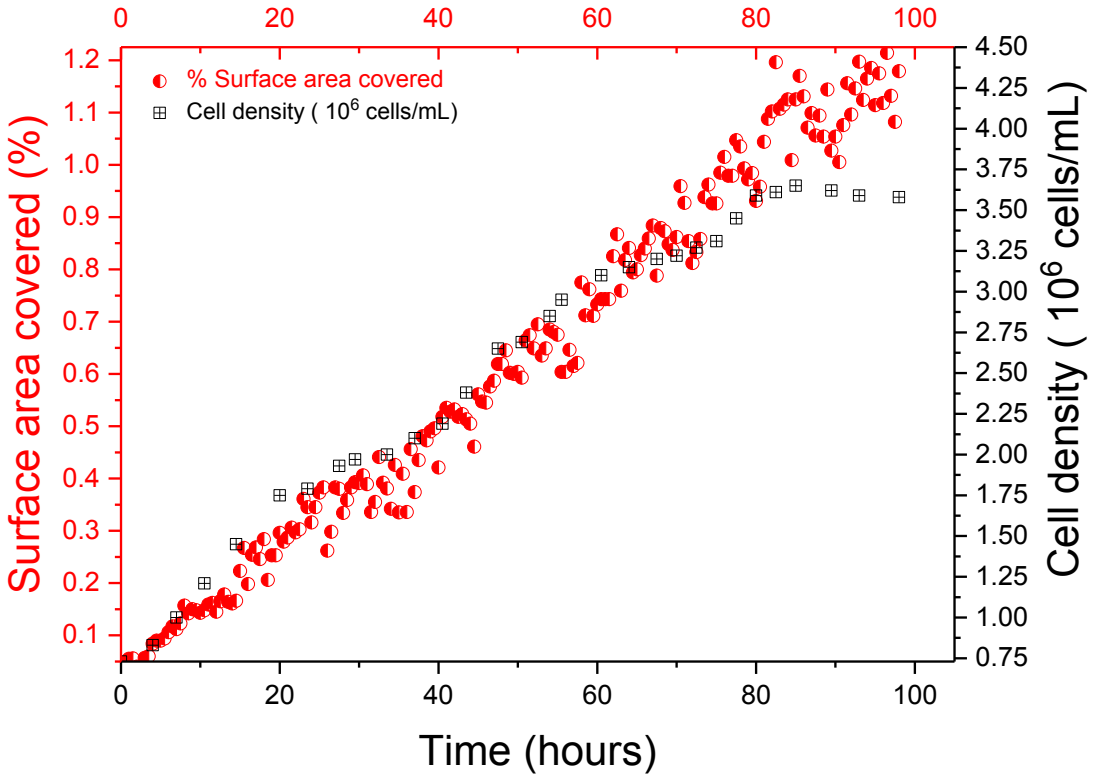


Figure 3-6 Correlation of cell density measurements determined using the developed image analysis protocol, expressed as % surface area covered (red/white circles), with measurements made manually using a haemocytometer over the assay period of 100 h (black crossed squares).

3.3.3 On-line removal of cells and particulates

Analysis using a SI-CE[17,18,23,24] system will require a cell- and particulate free sample to prevent clogging of the valve, capillary and fluidic connections. Filtration is an obvious choice, and in the CE-based bioprocess monitoring system reported by Turkia *et al*. filtration was used to obtain a cell- and particulate free sample from the suspension fermentation. Filtration, however, is prone to clogging, especially when used for extended periods of time, and Turkia *et al.* reported issues with cell agglomeration on the filter. H-filters exploit laminar flow to ensure the transport of small molecules between two parallel fluid flows based on diffusion differences between small molecules, macromolecules and particulates[25,26]. The concept was introduced by Yager *et al.*[27], and has been widely used in different designs and forms[28,29,30,31,32,33,34]. In this work, the use of an H-filter to feed a cell-free sample from a suspension culture into a sequential injection capillary electrophoresis instrument (SI-CE) was investigated.

A H-filter with a 40 mm long, 1 mm wide and 0.285 mm high central channel was made by soft lithography in PDMS using a dry film photoresist template created using a transparency mask. The low Reynolds number (Re<<1) dictates laminar flow, and hence transport between the two flows is only based on diffusion. Several models and tests were initially conducted to ensure the efficiency and effectivity of the device design. For example, 5 μm fluorescent beads were diluted and used to resemble cells where at the proper flow rate and channels designs they did not have the time needed to be diffused from the donor to accepter solution according to fluorimeter testing of products from each channel. Array reader and collecting flows measurements have been studied for the same purpose. The transport of the target analytes from the donor (media) into the acceptor (milliQ water) at flow rates ranging from 8 to 110 $\mu L.min^{-1}$ was quantified by connecting the H-filter with the SI-CE system discussed below. As illustrated in (Figure 3-7), the concentration of analyte in the acceptor phase was at

least 30% of the target donor phase at a flow rate of 8 μL.min^{-1} – the minimum flow rate the peristaltic pump could deliver (residence time in the H-filter 9.6 seconds).

Fluorescence microscopy confirmed Rhodamine-6G stained Jurkat cells did not cross into the acceptor solution whilst the small molecule fluorescein diffused into the acceptor solution (Figure 3-8).

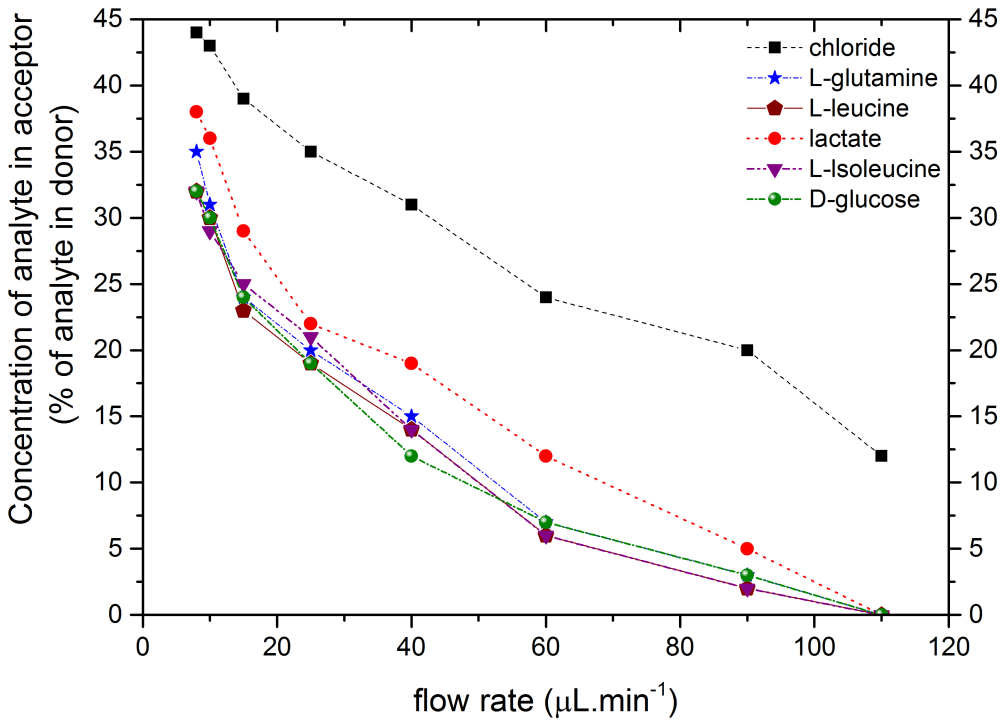


Figure 3-7 the influence of the flow rate on the transfer of analyte from the donor into the acceptor solution in the H-filter. The analyte concentrations in the acceptor solution were measured at different flow rates and expressed % of donor solution.

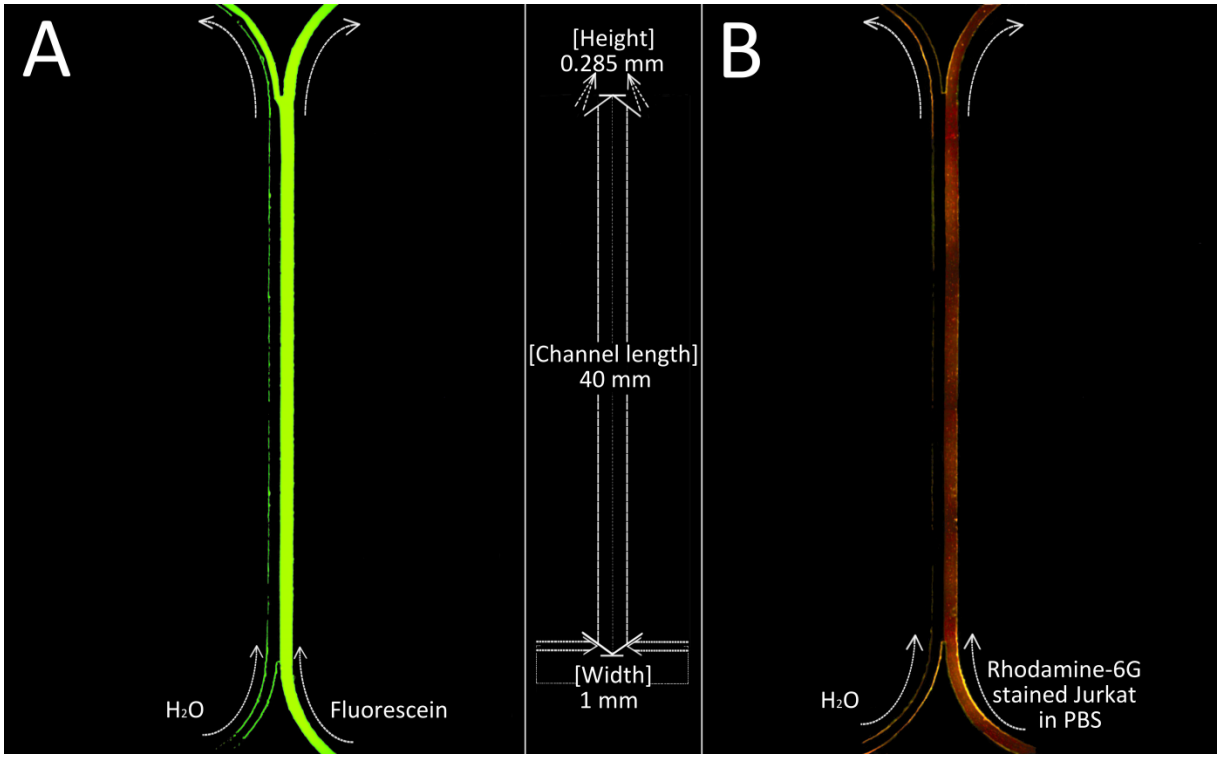


Figure 3-8 Visualising H-filter performance when operated at 8 µL.min^{-1} for fluorescein and Jurkat cells. A: Diffusion of 10 ppm fluorescein from the donor stream into the acceptor stream (water). B: Rhodamine-6G stained Jurkat cells do not diffuse into the acceptor stream (water) at 8 µL.min^{-1}.

3.3.4 SI-CE

Traditionally, sample injection in capillary electrophoresis, like in HPLC, is performed from a sample vial. As this complicates its use for online monitoring purposes, our group has developed SI-CE systems for rapid identification of inorganic ions in explosive devices[23] and water samples[17], demonstrating that the speed, selectivity and efficiency of CE could be used in conjunction with a flow-through interface. A similar system was used for monitoring lactate production in adherent cell cultures in chapter 2, demonstrating minimal sample use with 228 assays over 3 days using a total of 1.99 mL of sample[18].

The SI-CE system used for this work, connected with the H-filter cell counter, is schematically indicated in Figure 3-1, a more detailed schematic can be found in Figure 3-3. The system comprises of two pumps (for sample and BGE respectively), a 6-port valve to select sample or BGE to move towards the separation system and a T-interface connecting the 6-port valve with the 28 µm I.D. PMMA separation capillary and through a tubular ground electrode to the outlet vial. A two-way solenoid valve was positioned between the T-interface and outlet vial to direct the liquid into the separation capillary, allowing for flushing of the capillary and hydrodynamic sample injection. The control and data acquisition are fully automated and controlled using a PC with NI Labview. After filling the system with BGE, the 6-port valve is actuated to direct sample towards the T-interface. Brief closure of the two-way solenoid valve hydrodynamically injects a small sample plug into the separation capillary. With the solenoid open, the sample in the tubing is replaced with BGE, leaving only the small sample plug in the separation capillary. A high voltage (+30 kV) was applied for electrophoretic separation of the sample. The sequence of events was carefully optimized and the optimized conditions are detailed in Table 3-1. All separations were performed at ambient temperature (20 °C).

Combined with cell density, concentration profiles of the biomarkers glucose, glutamine,

leucine/isoleucine and lactate will provide insight in the cell metabolism and growth. With no previous report for the analysis of these analytes from cell culture medium by CE, a new separation method was developed. To separate glucose from other saccharides by CE requires a strongly alkaline BGE with a pH value of over 12 to dissociate the semi- acetal groups into their anionic form. The only used BGE for this purpose with conductivity detection was NaOH [35,36,37,38]. In our initial work, we developed a method based on the use of CTAB as a dynamic coating for EOF reversal and BGE consisted of 10 mM NaOH. 60 mM TEA and/or DEA were added to the BGE to lower the buffer conductivity in order to stabilize the separation baseline for better qualitative selectivity and accurate quantitative measurements. Even though good preliminary results were obtained in terms of low detection limits, low limits of quantification and high interday and intraday precisions values were unacceptable. Lactate and glutamine peaks could not be resolved from other components peaks in the RPMI-1640 media and the capillary coating failed when running the system for long time because the high content of protein in the media, that competed with CTAB binding sites on the surface of the capillary. However, many other approaches were explored for coating fused silica capillaries to achieve the required separation and stability against high alkalinity of the BGE. Polyelectrolyte coatings have shown promising results in terms of separation, selectivity and stability. Different polymers combinations were tested PDDAC/PSS/PDDAC, PDDAC/PEI/PDDAC, HDMB/PEI/HDMB, PDDAC/PEI/ HDMB and HDMB/PSS/HDMB, using different fused silica capillary internal diameters (10 μm I.D., 25 μm I.D., 50 μm I.D. and 75 μm I.D.), and applying diverse coating procedures and changing times for each layer coating, and changing various BGE solutions and concentrations around the required pH value of more than 12. This optimization process required a very long period of time since any change between any of the parameters mentioned required testing the coating stability for several days. However, using HDMB/PSS/HDMB coating, 75 μm I.D. fused silica capillary,

coating step time intervals of 5-10 minutes and BGE of (TEA/CAPS) gave the best results based on reproducibility of migration times and peak area, except that the stability of the coating could not last more than 20 hours when using diluted media. The slow dissolution of the silica at high pH was believed to be the source of the instability. After testing many alternatives, PMMA capillaries were shown to have stronger binding with the cationic polymers for more stable coating, and were used because of their superior stability compared to fused silica at high pH. Using a BGE consisting of 60 mM TEA and 10 mM CAPS and a capillary coated with a HDMB/PSS/HDMB polyelectrolyte coating all target analytes were well resolved from interfering compounds present in the media and a stable baseline was obtained (Figure 3-9). The performance of the optimized method was validated (Table 3-2).

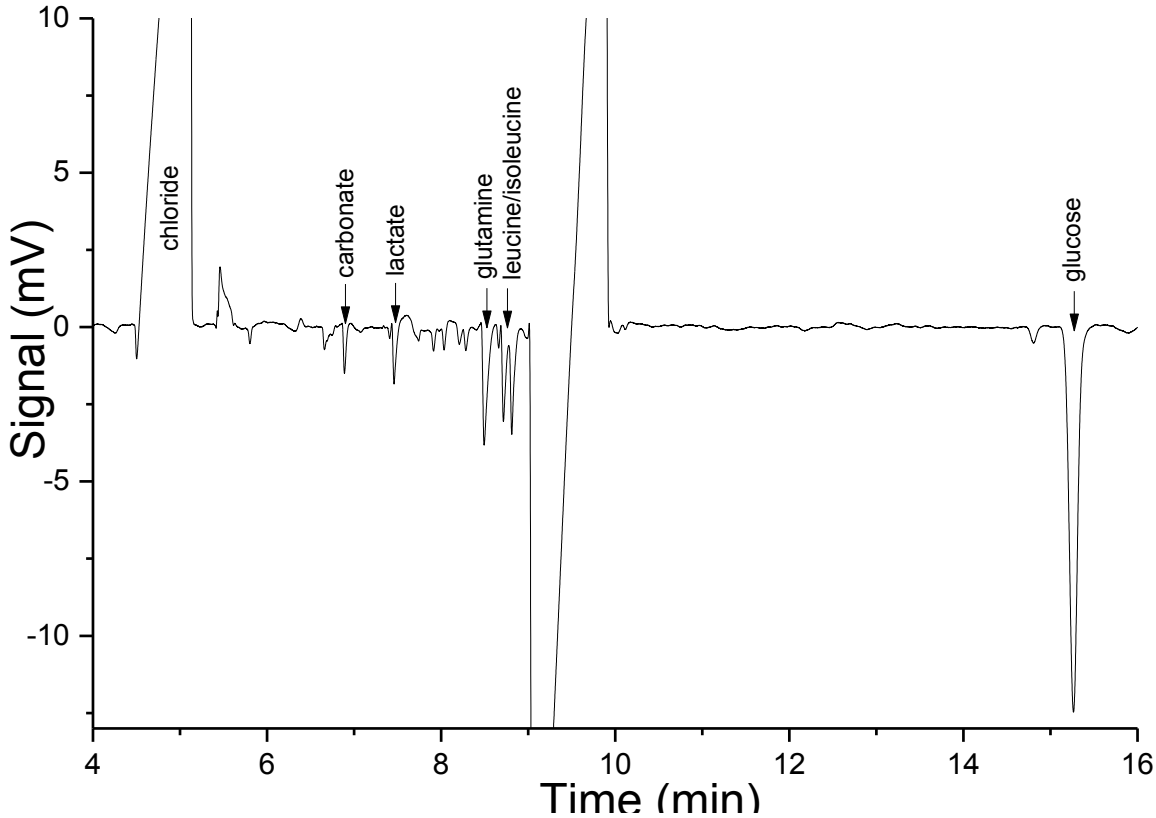


Figure 3-9 Electropherogram showing the target analytes, carbonate and chloride in RPMI-1640 cell culture medium. Conditions: 90 cm × 28 μm I.D. × 328 μm O.D. PMMA capillary coated with HDMB/PSS/HDMB; BGE: 60 mM TEA/10 mM CAPS, pH 12.4 with 0.075% PEI and 0.005% HDMB; +30 kV applied to outlet BGE vial while interface was grounded.

Table 3-2 Summary of validation results of the developed electrophoretic method in terms of repeatability, reproducibility, linearity and limit of detection and quantification, peak areas were corrected using chloride as internal standard.

	Glucose	Glutamine	Leucine/Isoleucine	Lactate
Intraday precision (electrophoretic mobility) n = 5	0.27%	0.15%	0.15%	0.12%
Interday precision (electrophoretic mobility), n = 5	7.21%	5.11%	5.07%	4.93%
Intraday precision (corrected peak area), n = 5	7.91%	4.57%	4.90%	3.85%
Interday precision (corrected peak area), n = 5	9.95%	6.99%	8.31%	7.21%
LOD	32 µM	25 µM	25 µM	17 µM
LOQ	50 µM	35 µM	35 µM	25 µM
Linearity (r^2, 0.15–5 mM)	0.9867	0.9906	0.9863	0.9912

Interday and intraday electrophoretic mobility precision averages were very good (5.58 % RSD and 0.17% RSD), an impressive achievement considering the system was not thermally controlled, and detection limits were obtained in the range of 0.17 μM – 0.32 μM. Because of the harsh separation conditions, difficulties in guaranteeing the stability of the polyelectrolyte coating over four days led to the decision to replace the capillary every day.

3.3.5 Bioprocess monitoring of suspension cells

Over a period of 4 days, 200 cell density measurements and 200 electropherograms were recorded using only 8.1 mL of sample, comprising around 4.5% of the total volume of the bioreactor. This cumulative volume over 4 days is smaller than the 8.94 mL of media per run required by Turkia *et al*. and could potentially be further reduced to zero by minimizing dead volume and recycling the donor solution back into the bioreactor after the H-filter. The concentration of glucose, glutamine, leucine/isoleucine and lactate is correlated with the corresponding cell density over the four-day period (Figure 3-10).

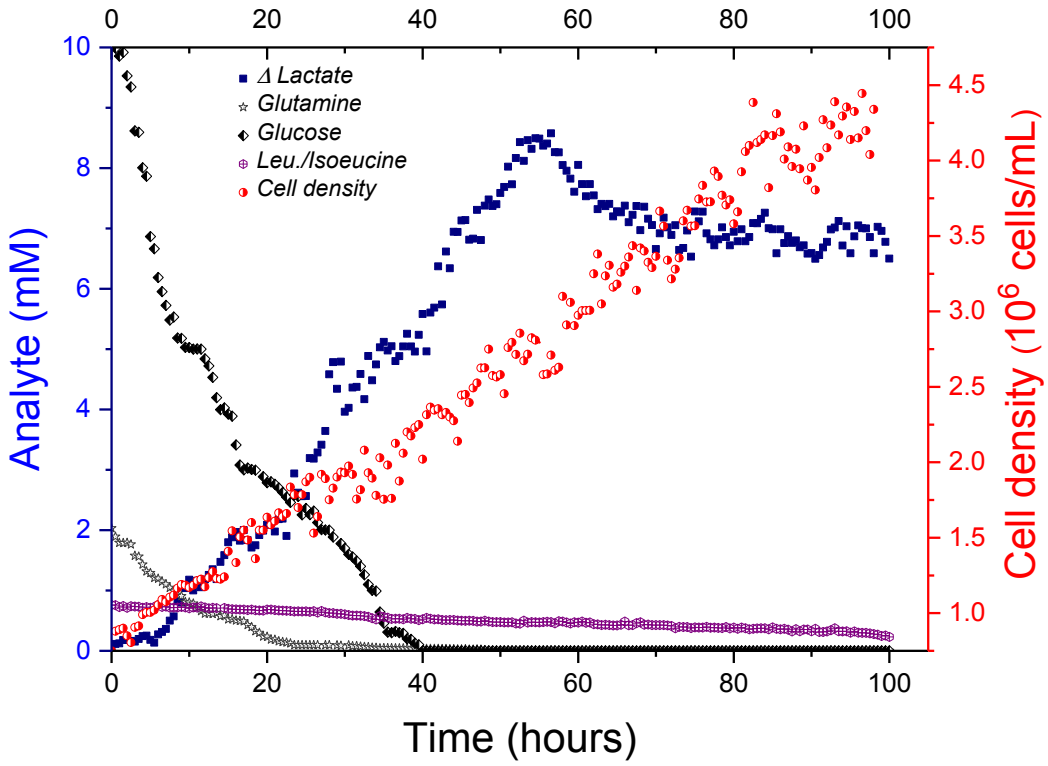


Figure 3-10 Monitoring of Jurkat cells over 100 hours, concentrations of glucose, glutamine, leucine/isoleucine, lactate on the left axis, cell density on the right hand axis. Peak areas corrected using Cl^- as internal standard. $\Delta_{Lactate}$ is the change in lactate relative to the basal lactate level in the media.

Three monitoring experiments were conducted spaced one month apart to ensure data could be compared between experiments. The average standard deviation was significant, ranging from 11-62% for lactate. When correcting for cell density (this was done every 30 minutes by dividing the amount of lactate produced per mL by the number of cells per mL, resulting in the amount of lactate produced per cell), however, this was reduced to 3-49%. The large standard deviations were typical for the earlier measurements, with the average RSD for lactate being reduced by a third from 24 to 16% when corrected for cell density. The raw and corrected data with error bars reflecting the standard deviation are presented in Figure 3-11. The RSDs <10% obtained for lactate for the last four measurements demonstrates the robustness of the presented system as the validation of the CE separation method indicates the analytical part already contributes 7.21 % of intraday variation (Table 3-2).

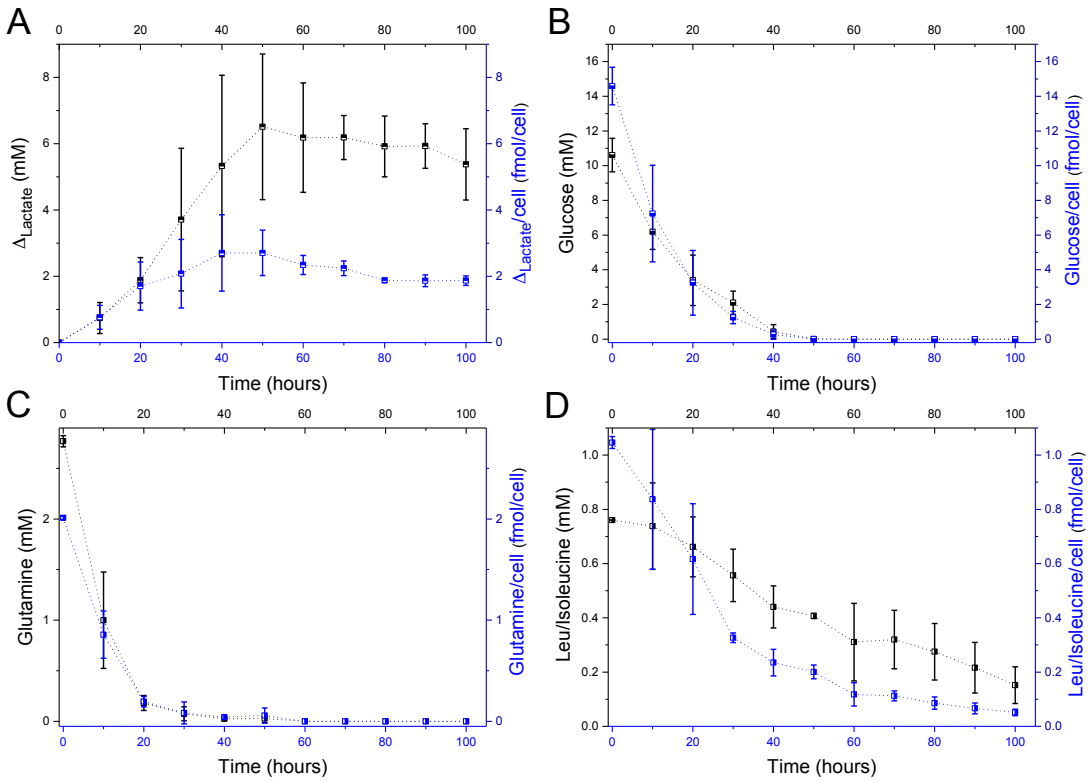


Figure 3-11 Averaged values with error bars for A: $\Delta_{lactate}$; B: glucose; C: glutamine and D: leucine/isoleucine for three 100 h experiments. Raw data (black) and data corrected for cell density (blue).

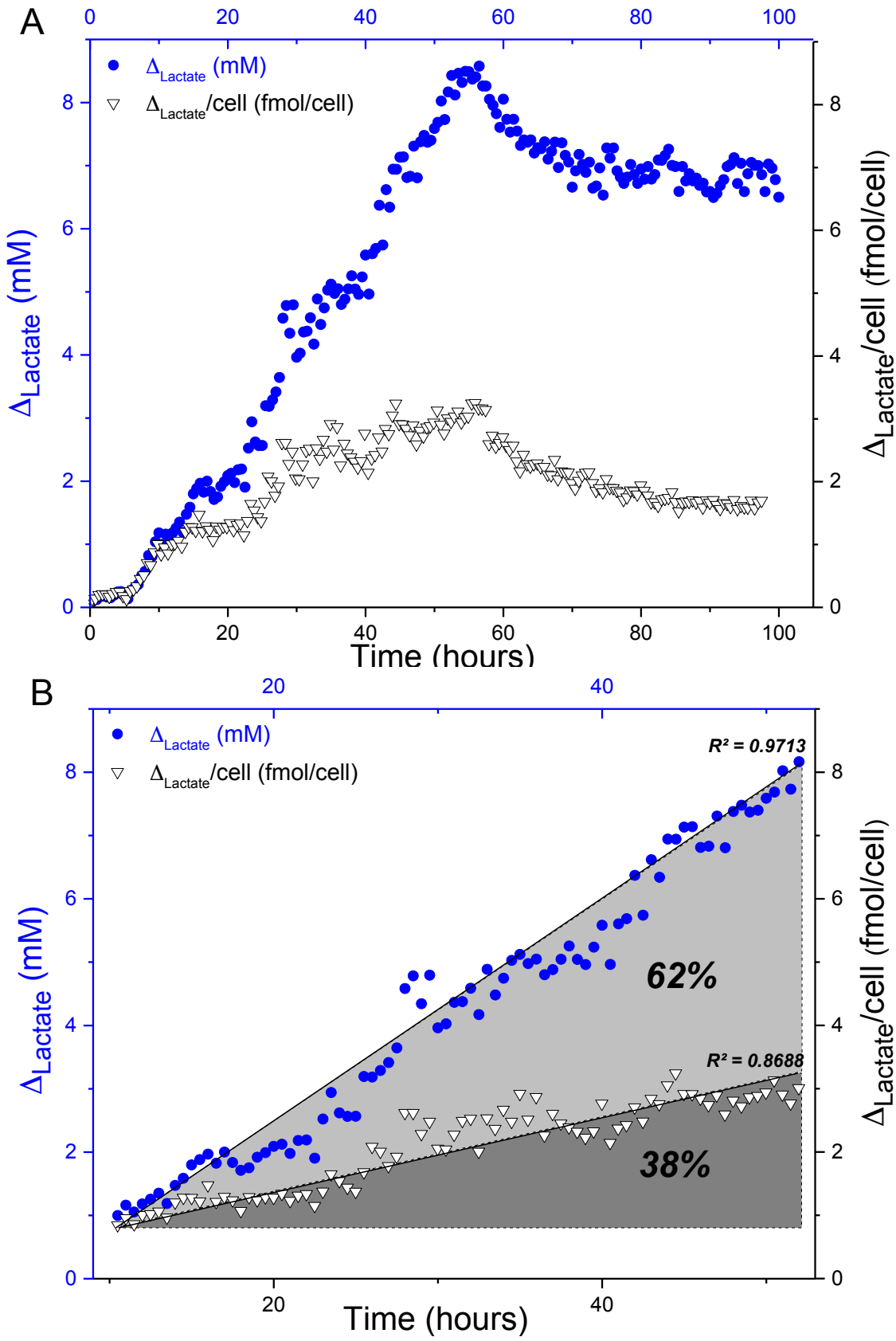
3.4 Discussion

Over the first 6 hours of culture, a considerable loss of glucose (44%) and glutamine (49%) was evident despite a low rate of cell proliferation and no change to lactate concentrations (Figure 3-10). The consumption of glucose is necessary for the cells to produce energy in the form of adenosine triphosphate (ATP), while the consumption of glutamine reflects glutaminolysis to produce nicotinamide adenine dinucleotide phosphate (NADPH) for anabolic processes, such as the synthesis of fatty acids, and protein synthesis. Both processes are essential for cell growth and duplication[39]. After 40 and 48 hours respectively, glucose and glutamine in the media were fully depleted while lactate levels increased steadily at a rate of about 0.17 $mM.hr^{-1}$ up to 52 hours. Interestingly, in the time period between 41 to 52 hours of cell culture – after all of the glucose and 99% of glutamine was depleted – cells kept growing at an almost unchanged rate. Autophagy might be one of the most probable explanations to maintain the energy required for cell growth. However, future studies should be conducted to investigate the detailed metabolic profile during this particular time period.

Between 52 and 90 hours of culture, a sudden change from lactate production to consumption was observed. This metabolic change is consistent with the need to explore other sources of energy to sustain growth under condition where all the glucose, glutamine and possibly other substrates have already been consumed. This shift from lactate production to consumption is characteristic of fast growing cells when the only remaining energy source becomes lactate itself[39,40]. From 90 hours to the end of the experiment at 100 hours, lactate levels decreased at a lower rate despite a continued constant cell growth rate, indicating an equilibrium between lactate production and consumption may have been established (Figure 3-10).

In order to correlate the metabolic markers to cell growth, a standardization of lactate concentration change with cell density over the entire culture period of 4 days was performed (Figure 1-12A). When zooming in on the time period from 10 to 52 hours (Figure 1-12B), a

linear correlation between the production of lactate and cell density was observed after the first few hours representative of the lag phase of cell growth. The corrected data elucidate that over this 10-52 h interval, 38% of lactate production was due to the increased cell metabolism as represented by increase in lactate production per cell indicated with the dark grey area, while 62% was a result of increased cell density (light grey area). Figure 1-12C reflects the interval between 52 and 98 hours. Whilst the lactate measurements indicate a stagnating increase in lactate production, the data corrected for cell density show a negative trend, indicating a change in metabolic state to where the cells consume lactate. Because the lactate consumption per cell remains constant over this period, one can conclude the measured increase in lactate consumption is predominantly due to increased cell density (Figure 1-12C).


A
0
20
40
60
80
100
Δ Lactate (mM)
Δ Lactate/cell (fmol/cell)
Δ Lactate (mM)
Δ Lactate/cell (fmol/cell)
Time (hours)
B
20
40
Δ Lactate (mM)
Δ Lactate/cell (fmol/cell)
R² = 0.9713
62%
R² = 0.8688
38%
Δ Lactate (mM)
Δ Lactate/cell (fmol/cell)
Time (hours)

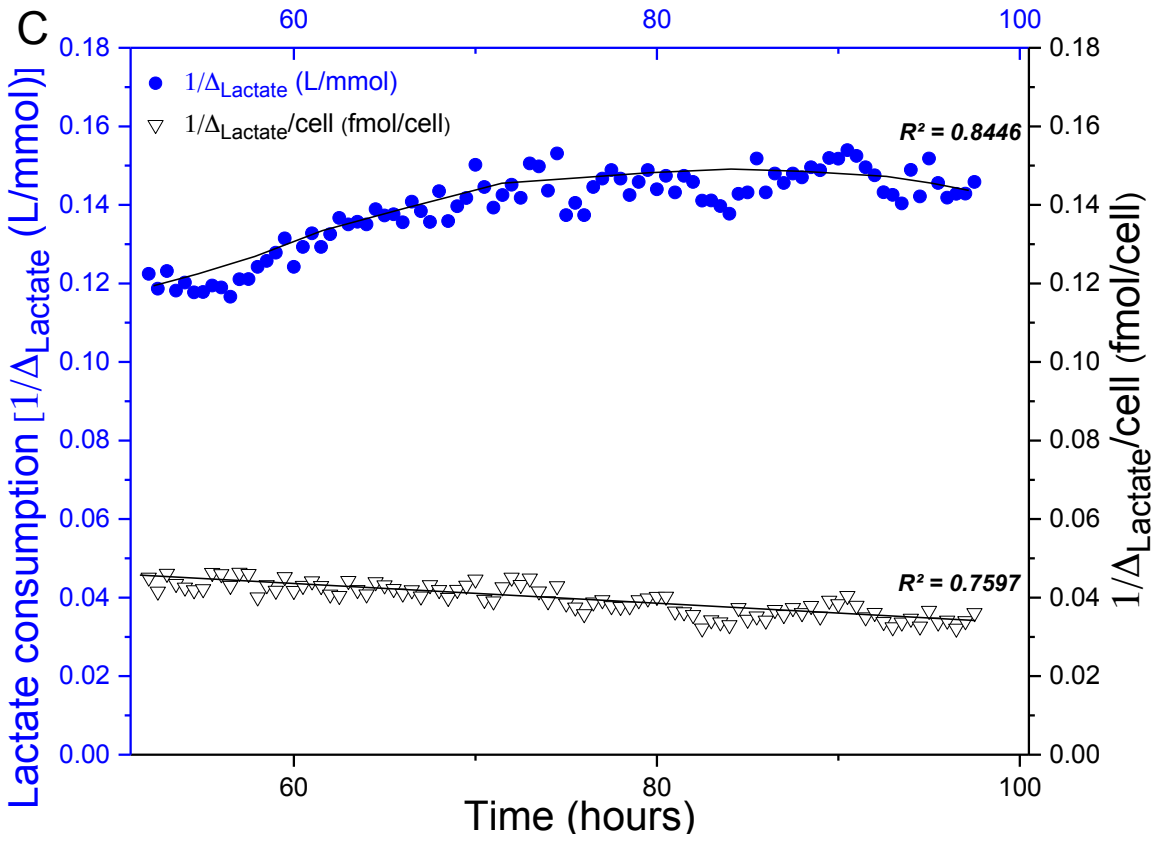


Figure 3-12 Standardization of changes in lactate deviation from the basal lactate level in the media by cell density, A: Comparison of $\Delta_{lactate}$ from 0-100 h expressed as raw data (solid cycles) and corrected for cell density (open triangles), B: Comparison of $\Delta_{lactate}$ from 10 - 52 h expressed as raw data (solid cycles) and corrected for cell density (open triangles), C: Lactate consumption ($1/\Delta_{lactate}$) (solid circles) and lactate consumption corrected for cell density (open triangles) from 52 - 100 h.

The detailed analysis of the lactate date provided above demonstrates that access to the time-dependent concentration and cell density changes provides the data required to generate a deeper understanding of the status of suspension cultures at any given time point, which will ultimately lead to a better control of bioprocesses. It is important to note that in this work we focused on key anionic nutrients and metabolites, but changes to only the separation chemistry would allow broadening the scope to analytes compatible with conductivity detection. Relatively small changes to the SI-CE hardware would increase the set of target analytes by at least a factor 2 without increasing the sample volume using a dual SI-CE system, as demonstrated by our group for online automated simultaneous separation of anions and cations in tap water[17]. Lastly, the system is compatible for coupling to a mass spectrometer, or to exchange the conductivity detector with a photometric unit for absorption of fluorescence detection. One should keep in mind the flow conditions for the H-filter presented here were optimized for small molecules, and would have to be re-optimised when targeting analytes with a significantly larger diffusion constant.

3.5 Conclusion

A new platform for online and automated monitoring of a mammalian suspension culture is presented capable of measuring cell density, nutrient- and metabolite levels every 30 minutes over a period of four days, using less than 41 µL of culture media per analysis. Digital imaging of the Jurkat cells in a microfluidic chamber provided an automated cell counting method with higher reliability than traditional cytometry at cell densities over three million cells.mL^{-1}. An H-filter was used to obtain a cell- and particulate-free solution that contained at least 30% of the target analytes. A 6-port valve was used to inject this sample into a sequential injection capillary electrophoresis system for quantification of glucose, glutamine, leucine/isoleucine and lactate. Data analysis revealed a metabolic change from lactate

production to lactate consumption at around 50 h, behaviour that is characteristic of fast growing cells. Correlation of the lactate profile with cell density provided insight in the metabolic state of the cells, allowing for delineation of the lactate production as a result of metabolism or cell density. The SI-CE method is flexible and allows for expansion of the analyte set without increasing the sample volume, making this new flexible platform a highly desirable asset to meet the future demands in process monitoring in the biopharmaceutical industry.

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Chapter 4

Parallel Monitoring of Pharmacological Assays using Sequential Injection Capillary Electrophoresis[4]

4.1 Introduction

Regulatory and ethical constraints on performing experiments on laboratory animals have directed pharmacological and toxicological studies towards cell-based based testing. Cell culture experiments can be conducted with high throughput on a wide variety of cell types, with data are typically derived as an end-point measurement using a plate reader. Whilst this is satisfactory for drug screening, pharmacological studies would benefit from more frequent measurements to obtain a better insight in the biochemical processes taking place over time. Ideally, changes in nutrient, substrate and/or metabolite concentration should be determined in an automated, low maintenance manner over short intervals and correlated with viability. The sample consumption over the duration of the cell culture experiment should be minimized, and the sterility of the cell culture should not be compromised by the analytical system. Current technologies available for chemical monitoring fail to meet the combination of these requirements. Sensors have good sensitivity and selectivity, but are through their specificity restricted to one analyte of interest[1] and as the signal is prone to drift require regular calibration[2]. To simultaneously analyse a range of analytes, high-resolution analytical techniques would be required. Spectroscopic techniques, for example using UV[3] FTIR[4] or RAMAN spectroscopy[5,6], can provide detailed chemical information but despite advances in

[4] Update from an article under review in, **Alhusban, A. A.; Breadmore, M. C.; Gueven, N.; Guijt, R. M., Parallel monitoring of pharmacological assays using sequential injection capillary electrophoresis,** *Analytical Chemistry***, 2016, ac-2015-04822f, xxx-xxx.**

the interpretation of spectra, resolving complex analyte sets with similar functional groups remains challenging. Mass spectrometry can be used to analyse cell culture media, but the extensive sample clean-up restricts its use for automated monitoring[7]. Chromatographic separations require extensive sample pre-treatment and each analysis takes >10 minutes, making it less suitable for a high throughput automated monitoring.

Capillary Electrophoresis (CE) is a powerful and fast separation technique capable of dealing with small sample volumes[8], and has been employed in bioprocess monitoring for broad range of analytes[9]. For example, Sandlin *et al.* employed a microfluidic CE system for *in vivo* bioprocess monitoring of amino acids through on-line coupling of the chip to a microdialysis probe[10]. Also using a microfluidic approach, the secretion of glucagon from pancreatic islets of Langerhans was monitored online using an electrophoretic heterogeneous immunoassay[11]. Turkia *et al.* developed a flow-through interface for a commercial CE instrument, which was used for monitoring organic acids over several days[12]. Our group developed a Sequential Injection CE (SI-CE) system comprising a low dead volume flow-through interface for automated sample injection[13,14], which we modified for the online analysis of lactate, glucose, glutamine, and leucine/isoleucine. Combined with an automated cell density measurement, the system was applied to monitor a culture of human T lymphocytes every 30 minutes over 4 days[15].

Here, we present a SI-CE system capable of monitoring five parallel cultures for pharmacological assays that are typically conducted at a number of different concentrations. This system is unique by, in addition to the CE separations, determining cell density of human T lymphocytes every 12 minutes over 4 days, using 60 μL of sample per analysis for five parallel cultures. To demonstrate the applicably of the system for pharmacological studies, the five parallel assays were conducted for three different pharmaceutical compounds selected based on their potential effect on lactate production. Being fully automated, flexible

and fast, this system can provide new perceptions to study biochemical and metabolic changes in pharmacological assays.

4.2 Experimental Section

4.2.1 Chemicals and Reagents

All chemicals were obtained as analytical grade reagent from Sigma–Aldrich, (Sydney, Australia) and used as provided unless indicated otherwise. All solutions were prepared in Milli-Q water (Millipore, Bedford, MA, USA) and micro-filtered prior usage. 20 mM lactate, 10 mM L-glutamine, 5 mM L-leucine, 5 mM L-isoleucine, 5 mM L-arginine and 2 M chloride standard solution were prepared and stored at 8 °C. Rotenone, β-lapachone and clioquinol (Ciba chemicals, Basel, Switzerland) all at 10 mM were prepared and frozen before dilution and addition to culture media. The cationic polyelectrolyte poly(ethylenimine) (PEI) (ACROS organics, Geel, Belgium) at 0.025% was added to BGE. An 80 cm polyelectrolyte coated fused-silica capillary was prepared from hexadimethrine bromide (HDMB) and poly(sodium 4-styrene sulfonate homopolymer) (PSS) and (HDMB) to reverse the EOF. The BGE consisted of a combination of tris(hydroxymethyl)aminomethane (Tris)) and N-cyclohexyl-2-aminoethanesulfonic acid (CHES) at pH 8.9.

4.2.2 Instrument Design

The SI-CE instrumentation is based upon a modified design in our laboratory[16]. It consists of two peristaltic pumps (PeriWaves, CorSolutions, Ithaca, NY, USA) for sample introduction and BGE delivery. A 7-port selector valve (MXP-7970, Rheodyne, Oak Harbor, WA, USA) was added to enable sequential sampling from each of the five culture flasks. A microfluidic chamber (1μ-slideVI$^{0.1}$, Ibidi GmbH, Martinsried, Germany) mounted on a microscope (Eclipse TS100, Nikon, Japan) was employed to determine the cell density. A PDMS microfluidic H-filter cast from an in-house made dry film photoresist template with a 40 mm

long, 1 mm wide and 0.285 mm high central channel was used to prevent cells and debris from clogging the injector valve. A three way solenoid valve (360T041SHH, NResearch, West Caldwell, NJ, USA) was used to control flow of either the extracted sample or the carrier solution into the injector. A high-pressure two-position six port switching valve (MXP9900-000, Rheodyne, Oak Harbor, WA, U.S.A) was used to deliver either BGE or sample to the capillary interface. A PEEK T-piece-connector (P-727, Upchurch Scientific, OakHarbor, WA, U.S.A.) was used to interface the flow injection system with the separation capillary. A fused silica capillary (50 μm I.D.; Polymicro Technologies, Phoenix, AZ, USA) was used for separation and its inlet was positioned in the interface to eliminate any carry over, with the outlet immersed in a glass vial filled with 25 mL BGE. A 20 mm long stainless steel syringe needle was used to connect the T-interface with Teflon tubing (1.5875 mm O.D., 0.508 mm I.D.) and used as hollow electrode that flowed to waste. A two way solenoid valve (HP225K021, NResearch, West Caldwell, NJ, USA) was used to stop the flow between the separation capillary and the waste tubing to perform a hydrodynamic injection. Detection was via a capacitively coupled contactless conductivity detection (C^4D) with optimized operational parameters as follow: (frequency, 2x high; voltage, 18 Db; gain, 200%; off set, 000). A high-voltage power supply (4300 Emco, Sydney, NSW, Australia) with reversed polarity was immersed in the outlet BGE vial. A NI USB-6212 data acquisition interface board (National instruments, Austin, TX, U.S.A.) using LabView v8.1 (National Instruments) was used to control the peristaltic pumps, selector valve, switching the solenoid valves and power supply, and for collection and saving the electrophoretic data.

4.2.3 Cell Density Measurement

A microscopy chamber (1μ-slide$VI^{0.1}$, Ibidi GmbH, Martinsried, Germany) prepared as described previously was placed on the stage of a phase contrast microscope connected to a digital camera (AM7023B Dino-Eye, New Taipei City, 241 Taiwan) to count cells by image

capture. Images were automatically captured at 40x magnification every 12 minutes as programmed in the LabView code to correlate with sample injection into the SI-CE. Images were analysed according to % surface area covered by cells or multicellular aggregates using the freeware package Image J to determine the cell density each run. Reproducibility and validation of this procedure is discussed elsewhere[15].

4.2.4 H-Filter

A microfluidic H-filter device was made by casting polydimethylsiloxane (PDMS) onto an in-house template. The H-filter exploits laminar flow with small molecules transport between the two parallel fluid flows based on diffusion. This was employed to acquire a cell- and particulate-free solution with at least 30% of the target analytes in the acceptor stream at a flow rate of 8 $\mu L.min^{-1}$. Design, fabrication, validation and extraction efficiency are described in detail in our previous work[15].

4.2.5 Instrument Operation

The SI-CE setup is schematically depicted in Figure 4-1 and was operated in 7 consecutive steps.

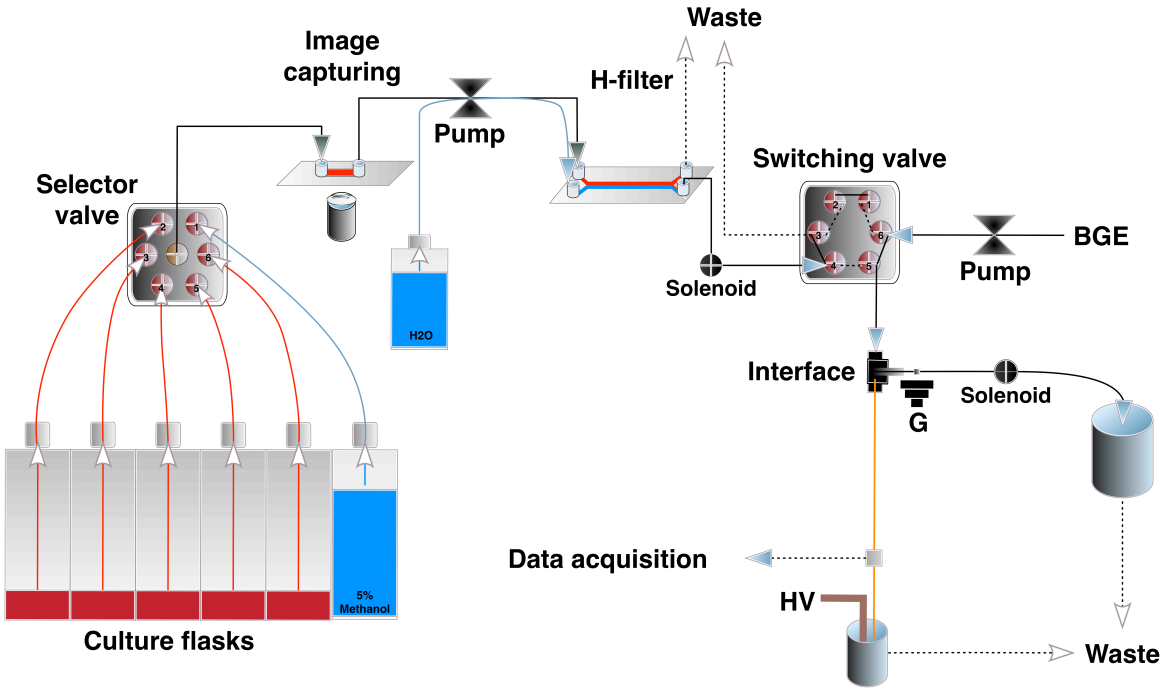

Figure 4-1 Schematic diagram of the experimental setup, the sample is withdrawn from one of the culture flasks, followed by cell density measurement using automated image analysis. In the H-filter, analytes are extracted into a cell-free sample solution which is analysed by SI-CE with C^4D. The selector valve ensures sampling from a different flask every run, with a run time of 12 minutes each flask is sampled once an hour.

The 7 port selector valve was switched to withdraw sample from the first culture flask at the lowest flow rate to enable the image capture of the cells inside the counting device while the three way solenoid was switched to flush the rest of previous sample to the waste. Simultaneously, the switching valve was switched back to direct BGE towards the T-interface at low flow rate while the two-way solenoid was open, and the high voltage (+28 kV) was applied on the capillary for the electrophoretic separation of the previous sample. The selector valve was switched back to withdraw the sample carrier solution at higher flow rate to carry the sample through the sample peristaltic pump into the H-filter. At the same time, the two-way solenoid was closed and both the T-interface and capillary were filled and flushed with BGE at higher flow rate to equilibrate the separation capillary, remove any air bubbles or partial blockage and stabilize the baseline. The sample flow rate was lowered back to 8 $\mu L.min^{-1}$ to enable the diffusion of target analytes from cells into an accepter solution in the H-filter. In parallel, the two-way solenoid was reopened and the flow of BGE reduced to eliminate the pressure in the T-interface. The three way solenoid was switched to transport the cell-free media into the switching valve at higher flow rate. The switching valve was switched to direct sample to the T-interface and the two-opening solenoid valve was closed for one second to inject the sample plug hydrodynamically into the separation capillary. In the final stage of this run, small amount of the carrier solution was withdrawn to initiate the sample pump for the next run. And in the meantime, the switching valve was switched to deliver BGE to clean the T-interface from the rest of the sample at a higher flow rate, and to prepare the setup to begin the next run. Events sequence, conditions and liquids volumes were cautiously optimized and are detailed in Table 4-1. All separations were performed at room temperature (20 °C).

Table 4-1 Sequence of events and volumes of BGE, sample and sample carrier solution used by the SI-CE system

	Operation	Time (s)	Selector valve position	Switching valve position	3-way solenoid direction to	2-way solenoid position	HV	BGE pump flow rate ($\mu L.min^{-1}$)	Sample & Carrier pump flow rate ($\mu L.min^{-1}$)	BGE used (µL)	Sample used (µL)	Carrier used (µL)
1	*Separation & Sampling*	452	2	1	Waste	Open	On	50	8	376.7	60.25	0
2	*Capillary flushing & carrying sample to H-filter*	115	1	1	Waste	Closed	Off	250	90	479.2	0	172.5
3	*Equilibrate*	2	1	1	Waste	Open	Off	0	0	0	0	0
4	*Extraction (H-filter)*	60	1	1	Waste	Open	Off	50	8	50	0	8
5	*Fill T-interface with sample*	78	1	2	Interface	Open	Off	0	180	0	0	234
6	*Hydrodynamic injection*	1	1	2	Interface	Closed	Off	0	10	0	0	0.2
7	*Fill T-interface with BGE*	12	1	1	Waste	Open	Off	250	8	50	0	1.6

4.2.6 Electrophoretic Conditions

Separations were performed using fused-silica capillaries (50 µm I.D., 360 µm O.D. and 80 cm in length, L_D = 70 cm). Capillaries were conditioned by flushing with 1 M NaOH at 0.5 $\mu L.min^{-1}$ for 10 min then Milli-Q water at the same rate for 5 min. For polyelectrolyte coating, the capillary was flushed with 1% aqueous solution of HDMB for 10 min at 0.5 $\mu L.min^{-1}$, Milli-Q water at the same rate for 5 min, 1% PSS for 10 min at 0.5 $\mu L.min^{-1}$, Milli-Q water at the same rate for 5 min, 1% aqueous solution of HDMB for 10 min at 0.5 $\mu L.min^{-1}$ and finally with BGE for at least 30 min. The BGE solution after optimization consisted of 35 mM of Tris (tris(hydroxymethyl)-aminomethane) and 35 mM CHES (cyclohexyl-2-aminoethanesulfonic acid) at pH 8.9. 0.025% (w/v) PEI was added for coating stabilization and selectivity alteration. Electrophoretic separations were conducted at -28 kV. A nitrogen gas line was connected to the BGE bottle to prevent change of the pH from the absorption of CO_2.

4.2.7 Cell Culture

The human T lymphocyte cell line (Jurkat ATCC® TIB-152) was cultured in T75 cell culture flask (Corning® 75 cm^2 rectangular canted neck cell culture flask with vented cap) routinely at 37 ºC and 5% CO_2 in a cell culture incubator, in RPMI-1640 Medium (23.8 mM $NaHCO_2$, L-glutamine without HEPES; 10% fetal calf serum; FCS, VWR, Murarrie, Australia). For the assays, 225 million cells were centrifuged at 200 g for 5 minutes at room temperature. Pellets were re-suspended gently in 200 mL culture medium and transferred into a 500 mL Erlenmeyer flask to form a total volume of 300 mL, equivalent to 7.5 x 10^5 $cells.mL^{-1}$. This 300 mL was distributed equally over 5 Pyrex® 250 mL Erlenmeyer flasks with screw cap (4985, Fisher Scientific, USA), adding 60 mL to each. These flasks were positioned and fixed in an orbital shaking water bath (OW1412, Paton Scientific, Victor Harbor, SA, Australia) at

37 °C and at 100 rpm. Water covered entire flasks to the neck to maintain a constant temperature through culturing time. The coverlid of each flask was drilled with a 0.95 mm diameter hole to insert (0.91 mm O.D., 0.19 mm I.D) Tygon tubing (SC0001T, ISMATEC, Wertheim, Germany) for sampling. All five tubing sections were connected to the five inlet ports of the selector valve at positions (no. 2 to 5). Position no.1 was connected to a carrier solution (5% methanol solution). A (0.91 mm O.D., 0.25 mm I.D) Tygon tubing (SC0002, ISMATEC, Wertheim, Germany) was connected to the selector valve outlet and to the inlet of a counting chamber positioned on a microscope connected to a digital camera (AM7023B Dino-Eye, New Taipei City, 241 Taiwan) for image capturing for cell density determinations. The sample peristaltic pump was connected to the outlet of the counting chamber. As careful sterility guarantee is very important to ensure reproducible and accurate outcomes the culture flasks, Tygon tubing with coverlids, peristaltic pump tubing and the H-filter were autoclaved prior their use. The whole setup was fitted in a laminar flow cabinet (ESCO, Singapore) to diminish the risk of contamination.

Serial dilutions with the required media were performed by precisely adding corresponding concentrations of each of clioquinol, β-lapachone and rotenone to its respective culture flask.

4.3 Results and discussion

Pharmacological studies are typically conducted at a number of different concentrations and include at least one or two blanks. As biological systems are dynamic, one of the ways to minimize biological variability is use multiple parallel flasks to culture simultaneously under identical conditions. This requires an analytical system for each individual flask, or the approach implemented here, a single analytical system that can sample from each flask in sequence. We used a SI-CE which is efficient, selective and provides rapid separations in an automated way that only requires a very small sample volume per analysis due to the use of a flow-through interface [13,14]. In our previous systems, only one culture could be monitored at a

time and sampling and SI-CE were conducted sequentially, leading to a significant loss in time. Here sampling and SI-CE are done simultaneously – while performing the separation for one flask, the next sample is being withdrawn, counted and treated at the same time. The run-to-run time was reduced from 22.5 min to 12 min (Table 4-1) which means that when monitoring 5 parallel cultures, each individual culture was analysed once per hour.

The time-based concentration profile of the metabolic biomarker lactate aids in understanding the effect of drugs on cell metabolism and growth. Since each cell culture contains a range of compounds including amino acids, sugars, proteins, vitamins, organic and inorganic ions, our previously developed method had to be optimised[16] to separate lactate from other ions. BGE pH and concentration, PEI concentration, capillary coating, I.D. and length were all examined. The optimised conditions were as follows: 80 cm x 50 μm I.D. (10 cm to detector) fused silica capillary coated with polyelectrolytes HDMB/PSS/HDMB in combination with a BGE containing, 35 mM Tris/35 mM CHES at pH 8.9 with 0.025% PEI.

4.3.1 Analytical performance of the multi-flask system

Evaluation of the system and chemistry was performed with a single flask of culture media over 4 days. The peak area for lactate was corrected using the chloride peak to eliminate any variation resulting from changes in injection volume. The method was validated, with precision of the corrected migration times of 0.71 % RSD (n=5) and 0.26% RSD (n=5) for interday and intraday respectively. The precision in normalized peak area was 7.88 % RSD (n=5) and 5.97% RSD (n=5) for interday and intraday. The LOD for lactate was 9 μM with a LOQ of 17 μM and the method was linear from 0.15–15 mM (r^2 = 0.9931) (Figure 4-2A).

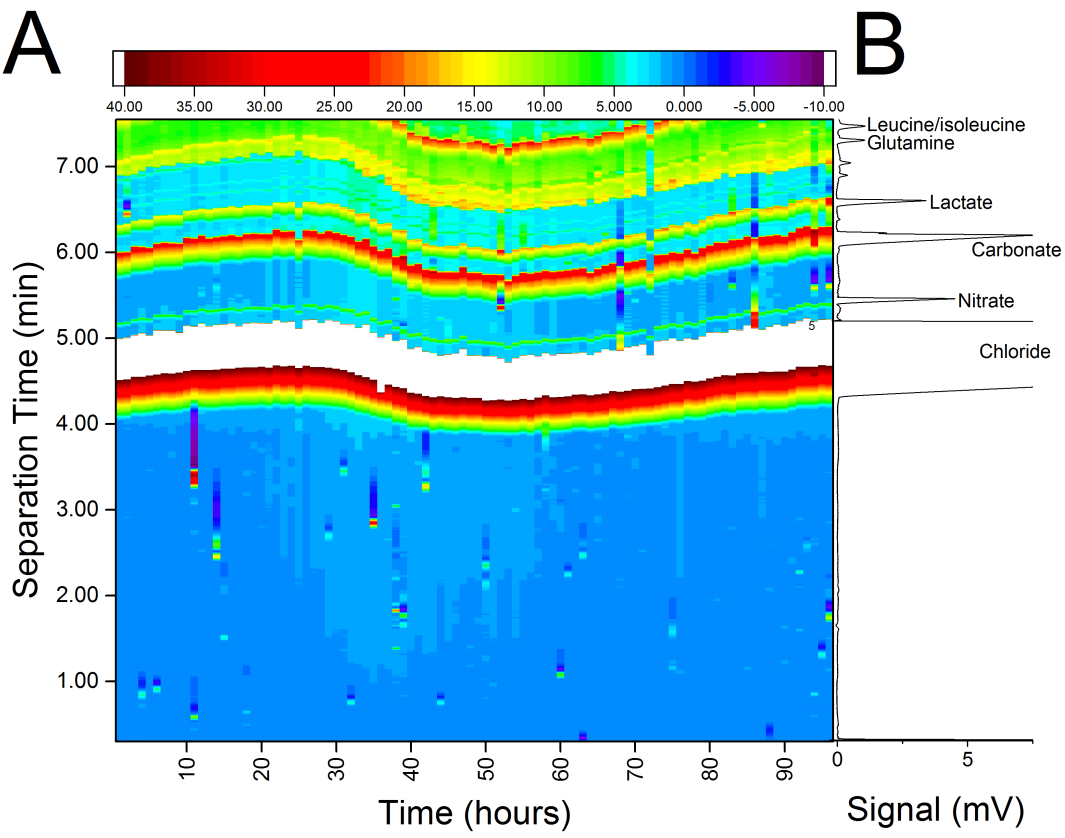

Figure 4-2 A: Reproducibility of analytes in RPMI-1640 cell culture media with 10% FCS (without cells) in one flask over continuous 4 days, B: corresponding electropherogram showing chloride, nitrate, carbonate, lactate and amino acids. Conditions: 80 cm × 50 µm I.D. × 350 µm O.D. fused silica capillary coated with HDMB/PSS/HDMB; BGE: 35 mM Tris/35 mM CHES, pH 8.9 with 0.025% PEI; +28

The same analysis was then performed by analysing the same culture media (without cells) in five flasks over 4 days, with the calculated lactate concentration of 1.37 mM, shown in Figure 4-3. It can be seen from the figure that there is excellent correlation between the lactate concentrations in each flask (1.37mM ± 0.119 in flask 1; 1.37mM ± 0.124 in flask 2, 1.37mM ± 0.109 in flask 3, 1.38mM ± 0.117 in flask 4, 1.37mM ± 0.110 in flask 5) (p = 0.765). The analytical variability between each flask across 4 days is less than 7.65%. It is important to note that because of our interest in cell metabolism and the common effect of the selected drugs on lactate, all data processing reported focuses only on lactate. Similar time-dependent profiles for glutamine and leucine/isoleucine are expected, demonstrating the richness of the data that can be obtained using this method (Figure 4-3B).

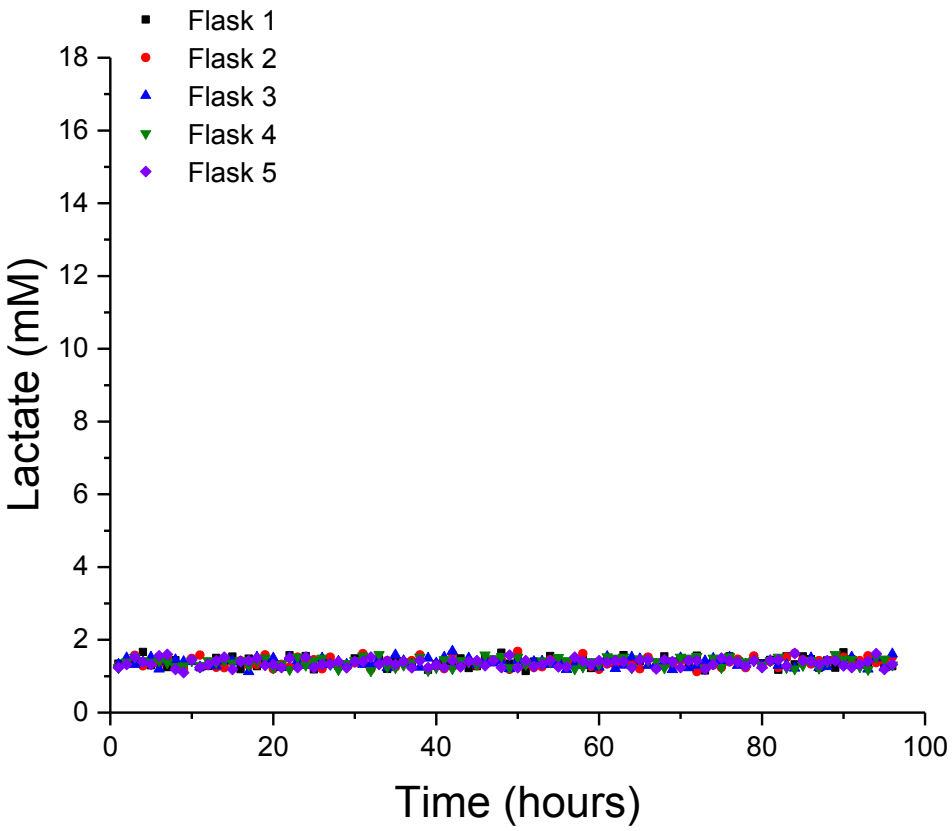

Figure 4-3 Reproducibility of lactate concentrations in RPMI-1640 cell culture media with 10% FCS (without cells) in five flasks over continuous 4 days.

4.3.2 Simultaneous monitoring of five parallel cell cultures

Biological systems are dynamic systems where the metabolic behaviour should be repeatable when the culture conditions are precisely controlled. In order to study the biological variability, 5 parallel cell culture flasks from a common culture of T-lymphocyte cell line (Jurkat) were cultured simultaneously under identical conditions and lactate data were correlated with the cell growth. Following the process described in Table 4-1, a sample was collected and analysed every 12 min. This resulted in a total of 480 electropherograms and 480 images for cell density quantification over 96 hr: 96 electropherograms and 96 images for each culture flask. Each individual analysis required only 60.25 μL of culture media, resulting in a total sample volume of 5.78 mL of media per flask over four days – less than 10% of the initial media volume. The change in lactate concentration for all 5 cultures over 4 days showed good similarity over the culture period (Figure 4-4). The combined variability here was 12.05%. This means that the biological variability is only 4.40% compared to 7.65% analytical variability. The cell growth, measured by the increase in cell density over time, also showed excellent similarity (Figure 4-5). Plots of the change in lactate concentration standardized with cell densities were also similar (Figure 4-6). The high level of similarity obtained for 5 cultures run in parallel provided the confidence that the developed system could be applied to study the effect of pharmaceutically active compounds known to influence lactate production. It is important to note that the incidence of infection/contamination was less than 1 in 15 flasks, as a result of careful optimization of the sterilization process. The sealing of the Tygon tubing in the coverlids with silicone was critical to working infection-free.

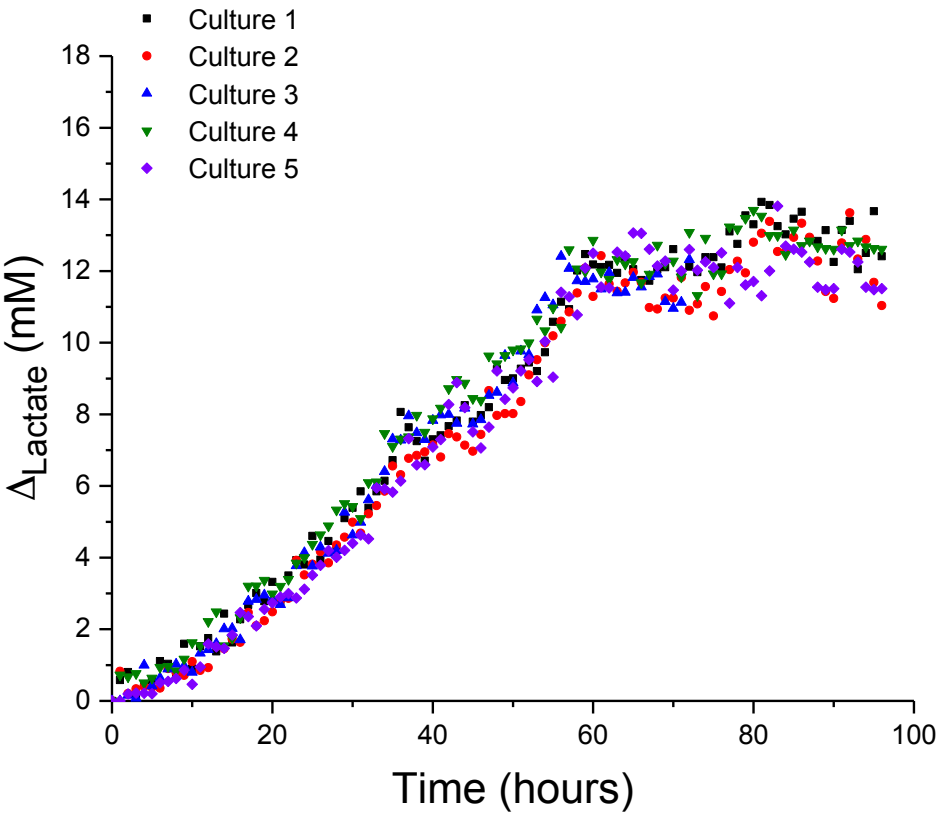

Figure 4-4 Simultaneous monitoring of 5 parallel cell cultures of Jurkat cells over 4 days, changes in lactate concentration.

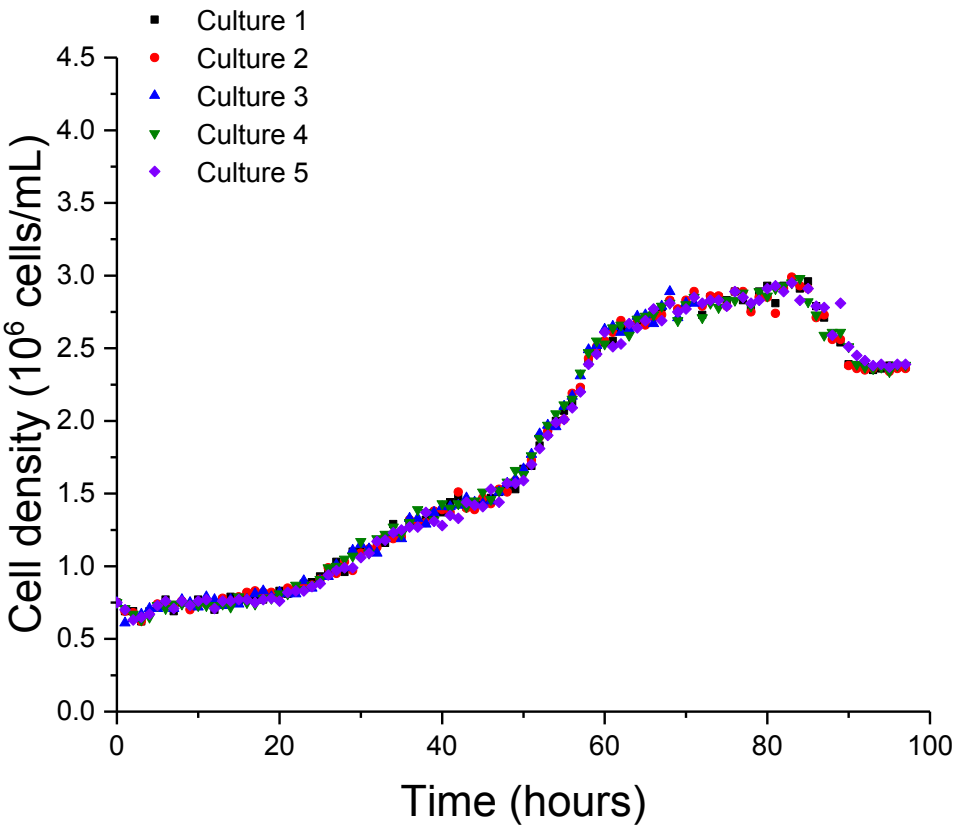

Figure 4-5 Simultaneous monitoring of 5 parallel cell cultures of Jurkat cells over 4 days, changes in cell density.

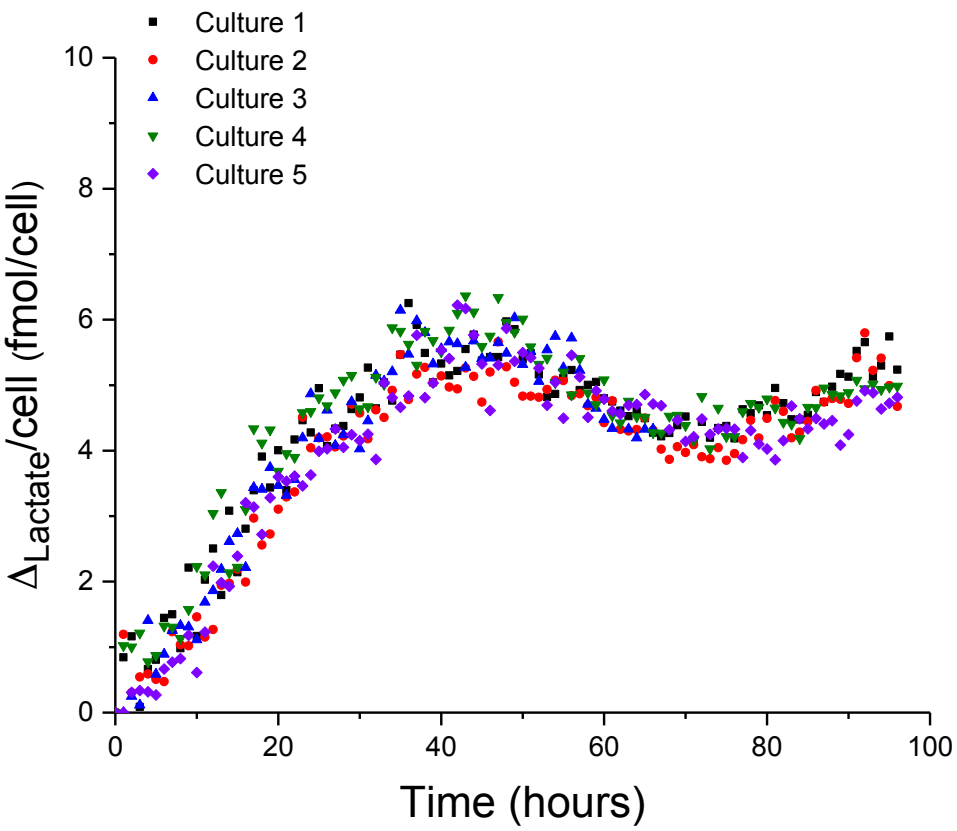

Figure 4-6 Simultaneous monitoring of 5 parallel cell cultures of Jurkat cells over 4 days, lactate concentrations standardized on cell density. No significant differences were found between the 5 parallel cultures based on the rate of lactate production per cell per time, (p values range 0.072 - 0.791), using one way ANOVA test.

4.3.3 Pharmacological Applications

The three drugs selected to demonstrate the SI-CE system for monitoring parallel suspension cultures all affect lactate production. Normally, lactate is produced from glucose and glutamine in mammalian cells[17] as a result of their conversion into ATP. Lactate is considered as one of the most important biomarkers of metabolism and energy status in mammalian cell culture[18]. Lactate levels are inversely correlated to mitochondrial function, as impaired mitochondrial function will enhance glycolysis in an attempt to meet the cells energy needs, which in turn relies on the conversion of pyruvate into lactate to generate nicotinamide dinucleotide NAD^+ required for glycolysis. This, together with the pharmaceutically active compounds, is depicted in a simplified schematic given in Figure 4-7. Mitochondrial impairment increases lactate production in Jurkat cells[19,20], whereas, enhancing mitochondrial oxidative phosphorylation decreases lactate production in tumour cells[21].

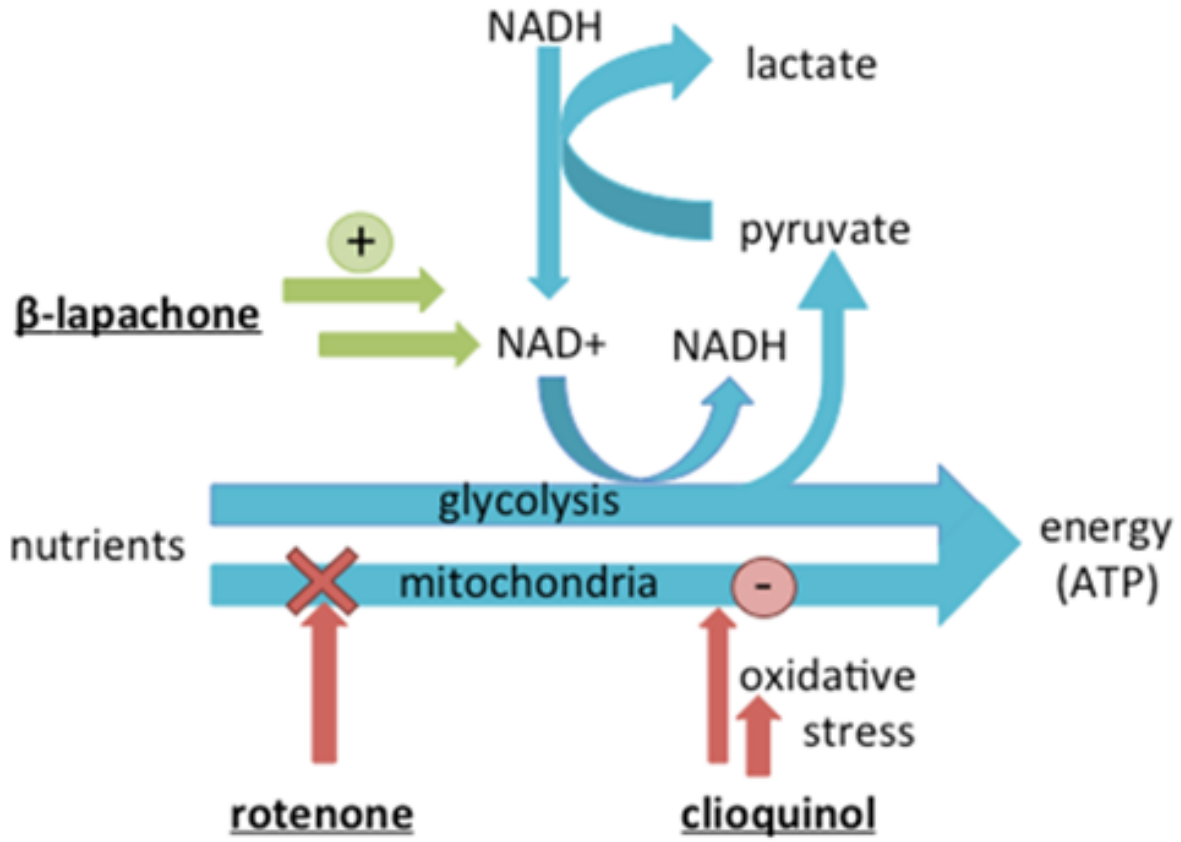

Figure 4-7 Schematic explanation of lactate production in cells as a result of the generation of NAD^+ from NADH to sustain glycolysis, rotenone is a mitochondrial poison, inhibiting mitochondrial function; clioquinol causes oxidative stress and as a result compromises mitochondrial function and β-lapachone results in an increase of NAD^+, which is believed to reduce the need for lactate production.

4.3.3.1 A Mitochondrial Inhibitor - Rotenone

Rotenone is utilized as an insecticide and its toxicity is based on inhibiting mitochondrial respiration[22]. Rotenone is a high affinity mitochondrial NADH dehydrogenase (complex I) inhibitor, an enzyme complex essential for mitochondrial oxidative phosphorylation[23] and at nM concentrations increases extracellular lactate levels[24]. Preliminary cell toxicity testing (data not shown) indicated a non-toxic concentration below 10 nM, hence three concentrations of rotenone were selected (1 nM, 6 nM and 10 nM) and control experiments were conducted in the other two flasks. While 1 nM and 6 nM rotenone increased lactate production (Figure 4-8), treatment with 10 nM rotenone decreased lactate production in comparison with the control flasks, which on face value, is inconsistent with the known behaviour. This effect was most pronounced in the time period between 5-40 hr. However, rotenone is also known to be cytotoxic and can cause cellular death[25], and the cell density profiles (Figure 4-9) confirmed a dose- and time-dependent reduction in cell growth for rotenone compared to controls. This demonstrates the importance of standardizing lactate production to cell density to fully understand the change in lactate metabolism. The standardized results (Figure 4-10) demonstrated lactate production increased with higher levels of rotenone, suggesting that mitochondrial activity was diminished.

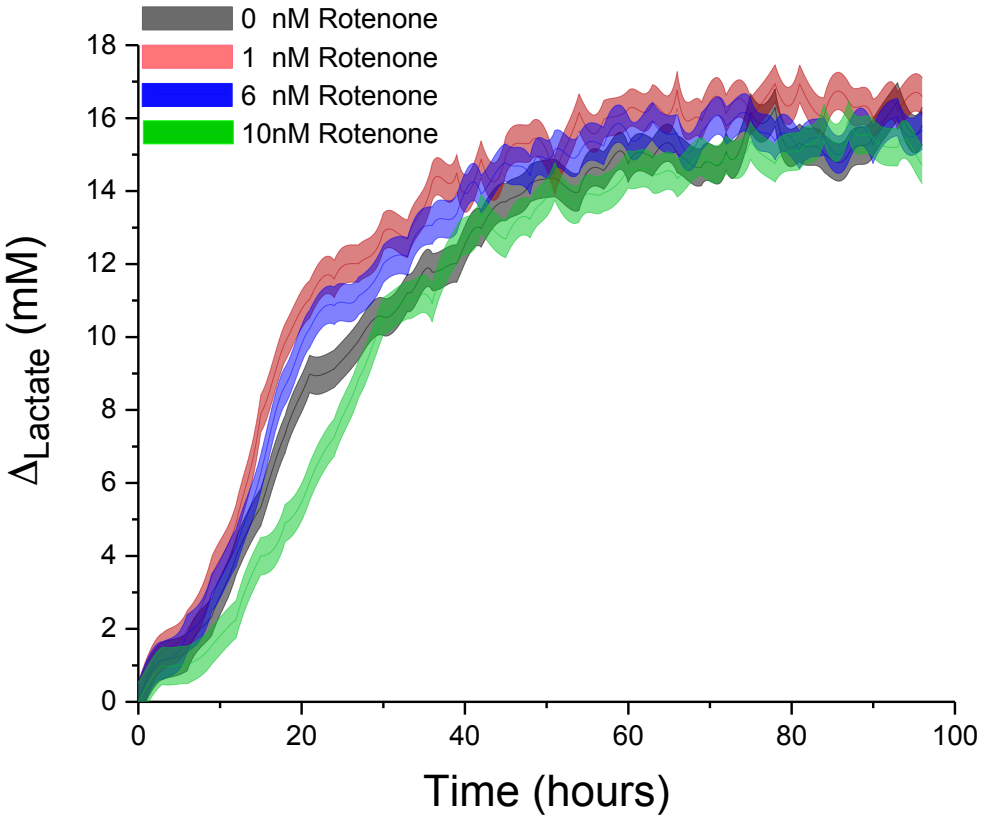

Figure 4-8 Monitoring of 5 parallel Jurkat cell cultures over 4 days in the absence or presence of rotenone, 1 nM, 6 nM, and 10 nM of rotenone was added to individual cell cultures (shown as lines with SD error-bars as coloured bands), changes in lactate concentration (n=5).

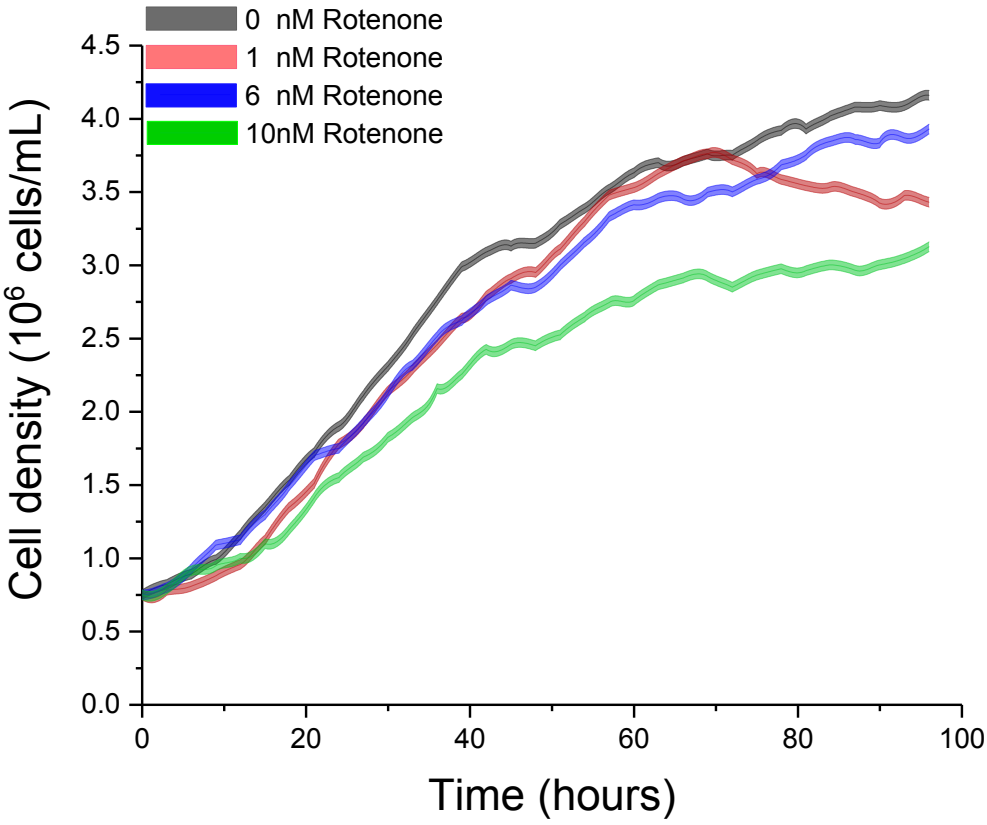

Figure 4-9 Monitoring of 5 parallel Jurkat cell cultures over 4 days in the absence or presence of rotenone, 1 nM, 6 nM, and 10 nM of rotenone was added to individual cell cultures (shown as lines with SD error-bars as coloured bands), changes in cell density (n=5).

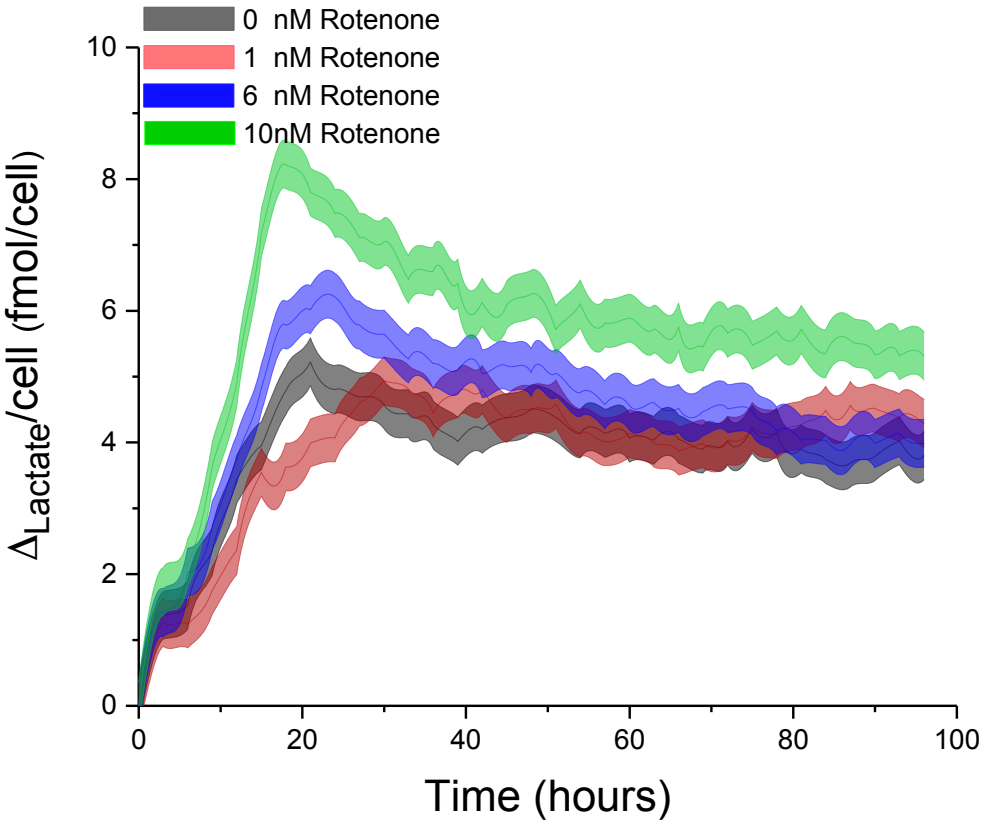

Figure 4-10 Monitoring of 5 parallel Jurkat cell cultures over 4 days in the absence or presence of rotenone, 1 nM, 6 nM, and 10 nM of rotenone was added to individual cell cultures (shown as lines with SD error-bars as coloured bands), changes in lactate concentrations standardized on cell density (n=5). Statistical significant differences, based on the rate of lactate production per cell per time, were obtained between 10 nM treatment and control ($p < 0.001$) and 6 nM treatment and control ($p = 0.046$), whereas no significant difference between 1 nM treatment and control ($p = 0.413$), using one way ANOVA test.

Treatment with 1 nM rotenone showed no effect, suggesting this concentration was below the effective inhibitory concentration. When looking in closer detail at the profiles, the additional information that can be obtained through monitoring rather than end-point measurements becomes clear. When looking at the measurement made 96 hr, only lactate production from the 10 nM rotenone treatment was distinctly different from the control and other concentrations ($p < 0.001$). When looking at the time-based profile, however, we can distinguish between the lactate levels obtained after 20 hr, and the rate of lactate production in the first 20 hr. For the 10 nM treatment, lactate was produced at 0.44 fmole/(cell.hr) with a significant difference compared to control ($p = 0.002$), for the 6 nM this was 0.31 fmole/(cell.hr) with a significant difference compared to control ($p = 0.029$), for the 1 nM 0.20 fmole/(cell.hr) which was not significantly different from the control 0.24 fmole/(cell.hr) ($p = 0.149$). After about 20 hr, the cell metabolism switched from lactate production to lactate consumption as evidenced by the stabilization and decrease of lactate produced per cell.

4.3.3.2 A mitochondrial toxin - clioquinol

Clioquinol (CQ) has been clinically used as an antibiotic and antifungal agent[26] before it was removed from the market due to neurotoxicity that was likely related to impaired mitochondrial function[27]. CQ induces mitochondrial dysfunction indirectly by increasing oxidative stress and lipid peroxidation (Figure 4-7). However, the cellular response to compensate for reduced mitochondrial ATP synthesis increases glycolysis similar to rotenone. Therefore, CQ treatment of cells was expected to increase lactate production comparable to rotenone. Three concentrations of CQ (1 μM, 5 μM and 10 μM) were added to three cell cultures, and together with two controls, these five flasks were monitored simultaneously under identical culture conditions. Lactate production (Figure 4-11) in controls and 1 μM CQ was similar ($p = 0.971$), and in contrast to the expectations decreased lactate production was observed for 5 μM and 10 μM CQ. A dramatic dose-dependent

reduction in cell density was observed compared to the untreated control cultures (Figure 4-12). The standardized lactate production (Figure 4-13) shows the expected dose-dependent lactate production. These results indicate that CQ has decreased the metabolic activity of Jurkat cells by impairing their mitochondrial function. In this case, by monitoring the culture, it is interesting to see that the lactate production rate is the same for the treated and control experiments, but in the CQ-treated flasks, there appears to be a correlation between the length of lactate producing phase CQ concentration. This may be explained by the fact the speed of change from lactate production to lactate consumption may be influenced by the cells affected by oxidative stress. The irregularity between 45 and 75 hr for the 10 μM CQ treated culture is most likely the result of the magnified effect of the increased imprecision with low cell count in the corrected values.

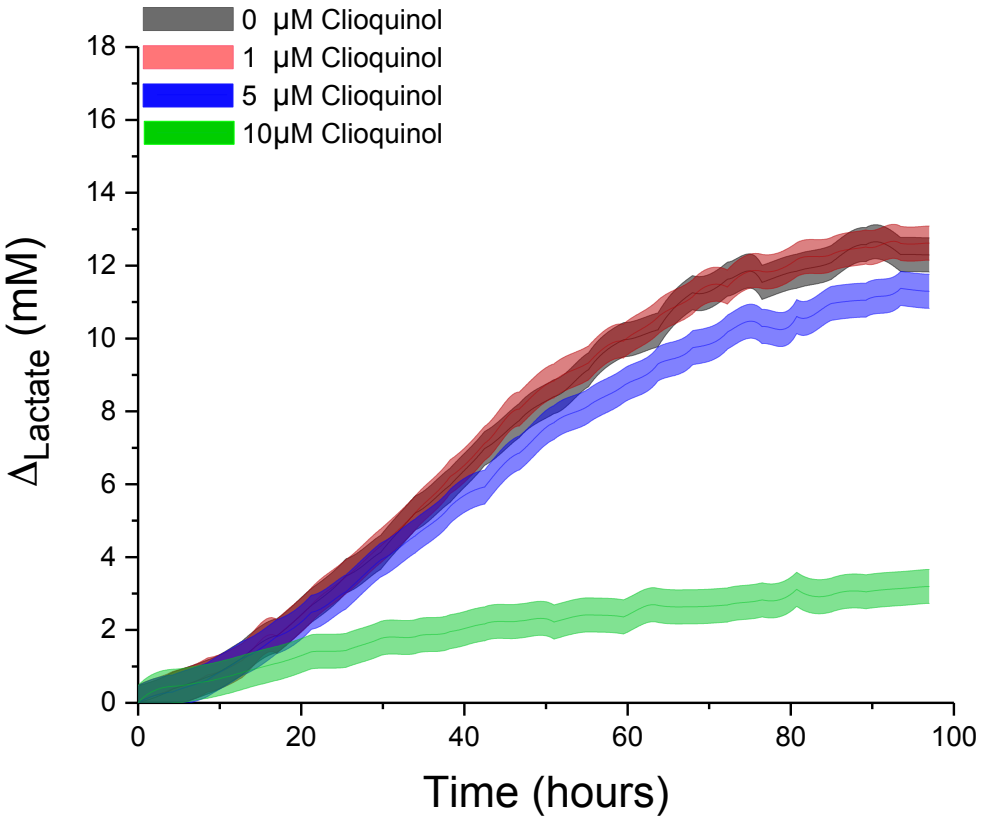

Figure 4-11 Monitoring of 5 parallel Jurkat cell cultures over 4 days in the absence or presence of clioquinol, 1 µM, 5 µM and 10 µM of clioquinol was added to individual cell cultures (shown as lines with SD error-bars as coloured bands), changes in lactate levels over time (n=5).

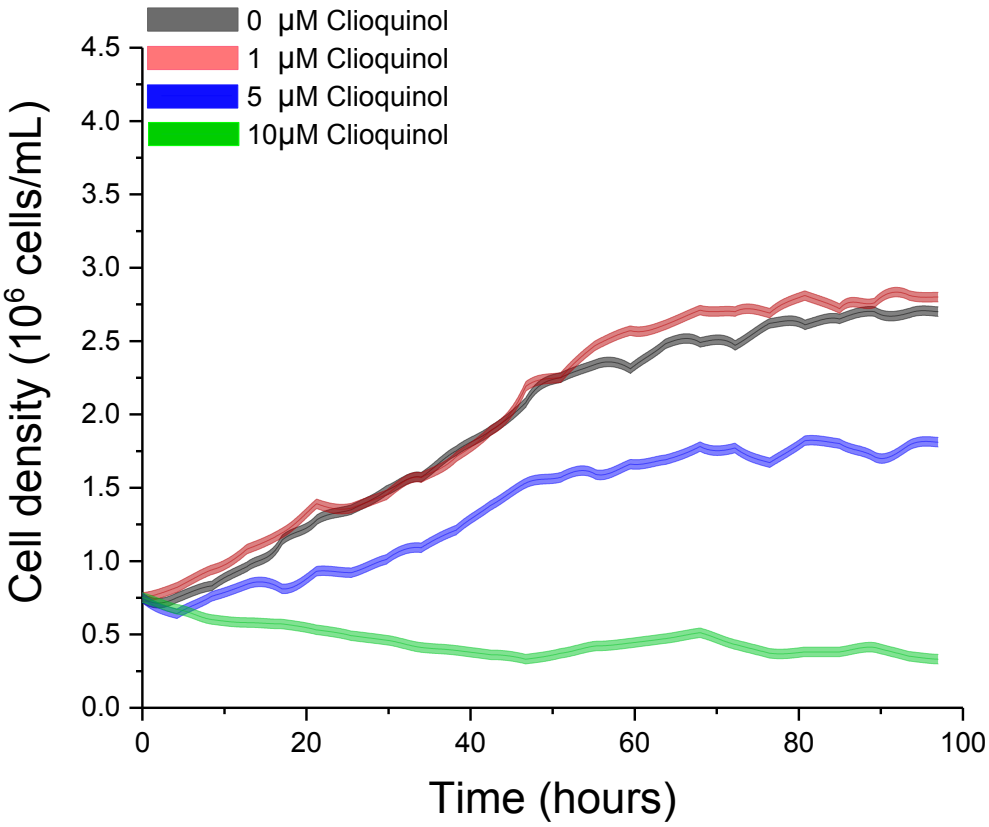

Figure 4-12 Monitoring of 5 parallel Jurkat cell cultures over 4 days in the absence or presence of clioquinol, 1 µM, 5 µM and 10 µM of clioquinol was added to individual cell cultures (shown as lines with SD error-bars as coloured bands), changes in cell density (n=5).

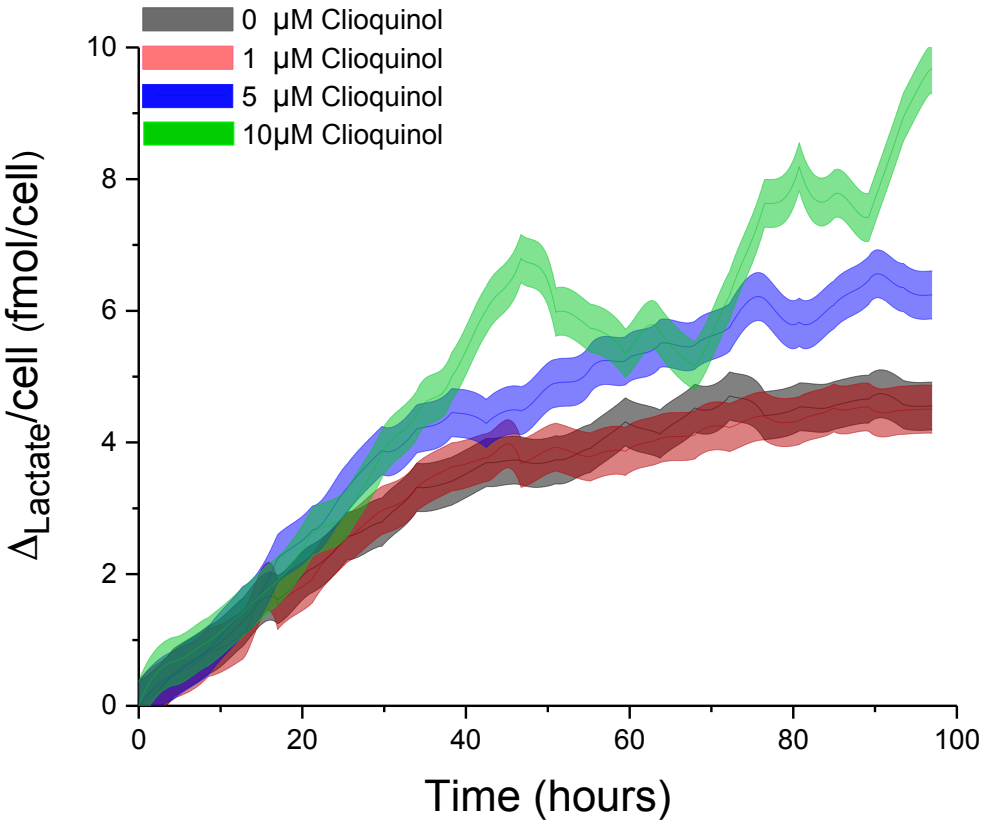

Figure 4-13 Monitoring of 5 parallel Jurkat cell cultures over 4 days in the absence or presence of clioquinol, 1 µM, 5 µM and 10 µM of clioquinol was added to individual cell cultures (shown as lines with SD error-bars as coloured bands), changes in lactate concentrations standardized on cell density (n=5). Statistical significant differences, based on the rate of lactate production were obtained between 10 µM treatment and control ($p < 0.001$) and 5 µM treatment and control whereas no significant difference was found between 1 µM and control.

4.3.3.3 A mitochondrial activated cytotoxin - β-lapachone

β-lapachone, a naphthoquinone derivative, increases the intracellular NAD^+/NADH ratio enabling the NQO1-dependent oxidation of NADH to NAD^+ [28]. Jeong *et al.* reported a reduction in lactate production by 1 μM β-lapachone in two cybrid cell lines[29]. The reason for reduced lactate production is thought to be partially a consequence of increased NAD^+ levels by β-lapachone, alleviating the need for NAD^+ production through the conversion of pyruvate into lactate (Figure 4-7). The previously reported effective β-lapachone concentration (1 μM) resulted in significant cytotoxicity in our cell line, with normal cell growth only observed at a dose of 0.2 μM or below. Therefore, β-lapachone was tested at 0.05 μM, 0.1 μM and 0.2 μM. The only significant difference in lactate levels was found between 0.1 μM treatment and control ($p = 0.015$) from the three concentrations (Figure 4-14). There was no dose-dependent effects were observed on cell density (Figure 4-15). No statistically significant differences in lactate production standardized on cell density (Figure 4-16) were obtained based on the rate of lactate production per cell per time between 50 nM and control ($p = 0.714$), between 100 nM and control ($p = 0.7644$) and between 200 nM and control ($p = 0.145$), using one way ANOVA test. This suggests β-lapachone does not affect the lactate production, and that the previously reported decrease in lactate production may have been the result of a decrease in cell density as a result of toxicity rather than an actual effect on lactate production[29]. This again clearly demonstrates the value of our system by being able to measure metabolite and standardize on cell number.

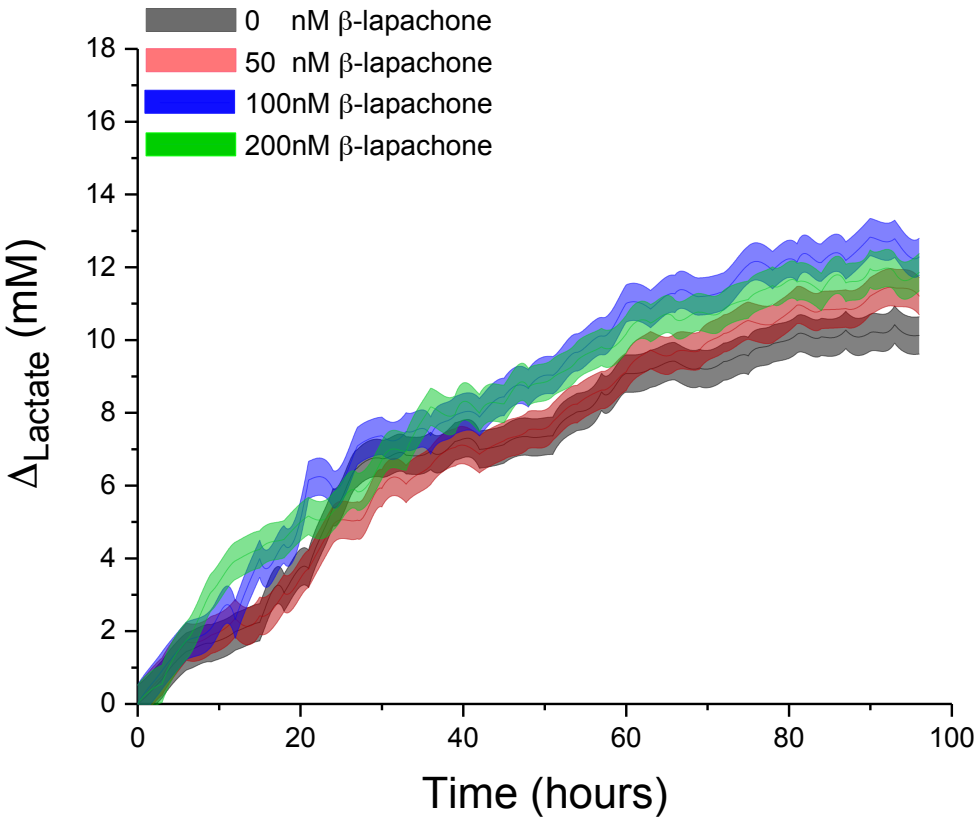

Figure 4-14 Monitoring of 5 parallel Jurkat cell cultures over 4 days in the absence or presence of β-lapachone, 50 nM, 100 nM, 200 nM of β-lapachone was added to individual cell cultures (shown as lines with SD error-bars as coloured bands), changes in lactate levels over time (n=5).

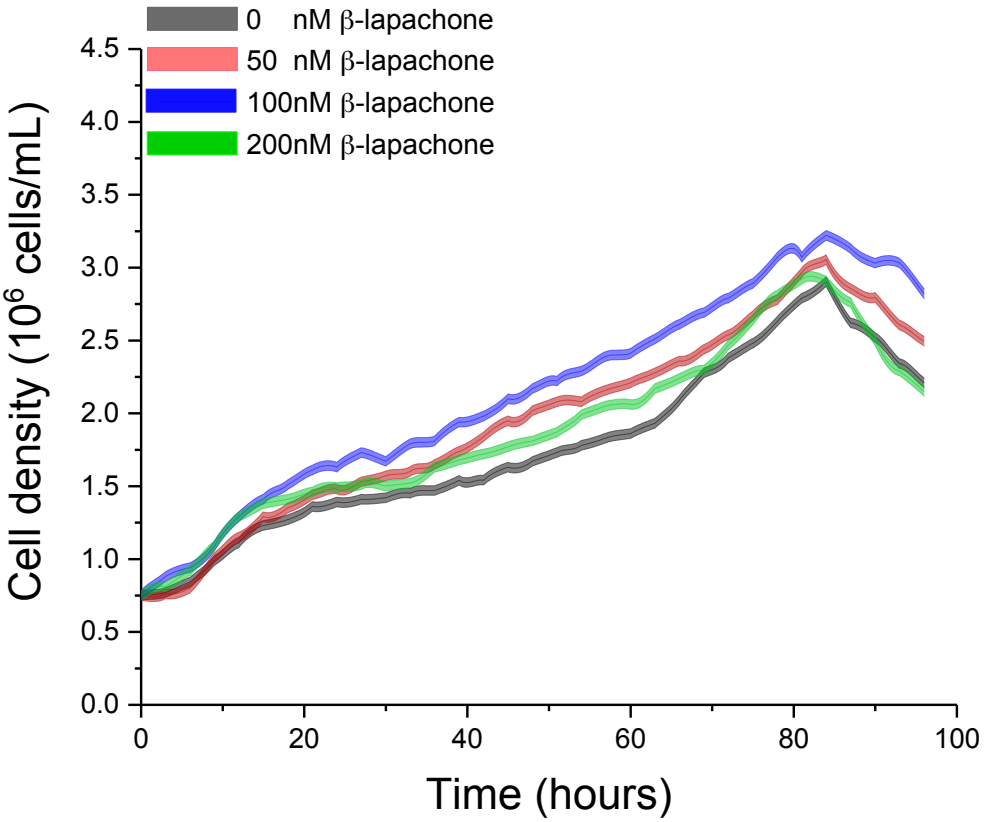

Figure 4-15 Monitoring of 5 parallel Jurkat cell cultures over 4 days in the absence or presence of β-lapachone, 50 nM, 100 nM, 200 nM of β-lapachone was added to individual cell cultures (shown as lines with SD error-bars as coloured bands), changes in cell density (n=5).

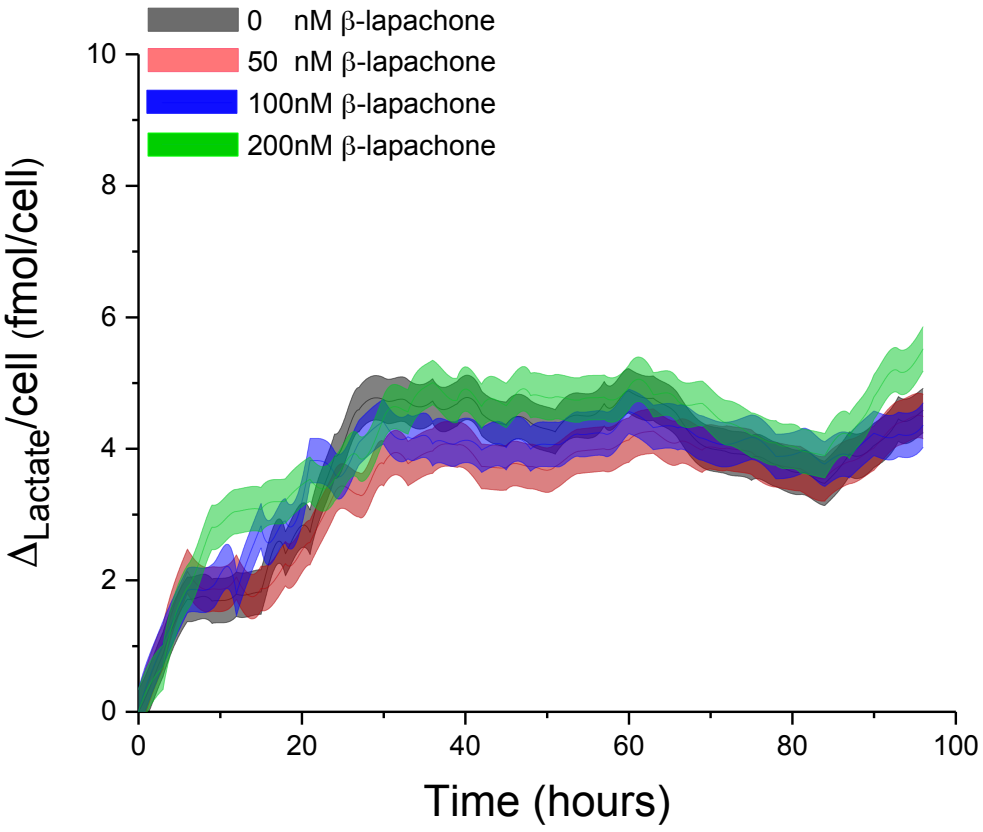

Figure 4-16 Monitoring of 5 parallel Jurkat cell cultures over 4 days in the absence or presence of β-lapachone, 50 nM, 100 nM, 200 nM of β-lapachone was added to individual cell cultures (shown as lines with SD error-bars as coloured bands), changes in lactate concentrations standardized on cell density (n=5). No significant differences were found between treatments and control based on the rate of lactate production.

From the three tested drugs, we can conclude that the presented SI-CE system for monitoring 5 parallel suspension cultures is suitable for conducting cell assays over at least 96 hr. The time-dependent profile provides deeper insight in the pharmacodynamics of the administered compound, especially because the measured effects vary over time. Recognizing further studies are required, the differences in impact on metabolism over time for rotenone and clioquinol may be indicative of their very different pharmacodynamics behaviour.

4.4 Conclusion

A fully automated system for monitoring variations in lactate concentration and cell density in five parallel cultures was developed and applied to test the effects of three different pharmaceutically active compounds. Integration of microfluidic units to a SI-CE system enabled cell counting and extraction of analytes into a cell-free sample. With a run-to-run time of 12 min, low sample consumption (60 µL/analysis), high throughput (480 analyses in 4 days), this robust system provides a new approach to study time-resolved pharmacological and metabolic mechanisms. In the study presented here, the system was applied to monitoring lactate, with the interday precision of the electrophoretic mobility and peak area 0.71 % RSD and 7.88 % RSD respectively. Cell density and lactate measurements correlated well across parallel cultures. A concentration-dependent increase in the production of lactate was observed for the drugs rotenone and clioquinol, with their pharmacological differences reflected in the lactate production rate over time. For rotenone, a steep increase was observed over the first 20 hr, whereas for clioquinol the effect was most noticeable over the 20-96 hr interval. For β-lapachone no concentration effect on lactate production could be demonstrated, reinforcing the importance of correcting biological effects for cell density.

With the target analytes defined by the CE separation, the proposed system provides a flexible platform for small molecule analytical targets, where targets can be changed by changing the separation chemistry with no or minimal changes to the hardware. Integration of

the cell count and H-filter devices, as well as further miniaturization of the system would lead to increased throughput.

4.5 References

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Chapter 5

General conclusions and future directions

1.1 General conclusions

Bioprocess monitoring is of high and increasing importance. First, in the production of biopharmaceuticals, monitoring will enable control of biological process parameters to warrant high product quality, fulfil regulatory requirements and prevent economic losses. Adequate monitoring tools will first provide the data required for improved understanding of these critical parameters within the dynamic biological processes, and then allow the processes are operated within the specified margins. Second, early phase of pharmacological studies increasingly rely on *in vitro* methodologies for compound testing and screening. Monitoring would enable replacement of current end-point assays with rich, time-resolved data providing insight in both the process and end-point. In the context of this thesis, novel contributions have been made in the context of bioprocess monitoring relevant for both production and drug screening. Sequential Injection Capillary Electrophoresis (SI-CE) combines a high resolution analytical separation by capillary electrophoresis with a flow-through interface for automated sample injection. Factors including automation, high sample throughput, low limits of detection, low sample consumption and the ability to out-scale make SI-CE attractive for on-line monitoring of bioprocesses.

In Chapter 2, an automated online system for monitoring lactate in different mammalian adherent cell cultures was presented. To sample the media, a sampling interface was developed for monitoring a human embryonic kidney cell line (HEK293) allowing for the injection of cell-free media into the separation system. The sampling interface comprised of a 500 μm hole drilled 500 μm from the end of 500 μm I.D. Teflon® tubing. Positioning of the

end of the tubing on the bottom of culture flask allowed for sampling of media 500 μm from the bottom, away from the region where the adherent cells are present. Minimising the dead volume in the system, the sample consumption per run was only 8.73 μL resulting in a total sample volume of 1.99 mL (less than 10% of media) used to monitor lactate production every 20 minutes over three days. The system was applied to the study the metabolic activity of mouse fibroblasts from a mitochondrial gene ND4 knockout cell line. ND4 is a subunit of mitochondrial respiratory chain complex 1, and the compromised mitochondrial function was expected to increase lactate production. Increased and accelerated lactate production was confirmed in the ND4 knock out during an initial experiment, however, these results could not be repeated using fibroblasts obtained from another mouse.

With biopharmaceutical research and production dominated by suspension cultures, the system described above was modified for suspension cultures in Ch 3. Three significant changes were made, i) a microfluidic device with digital microscopy unit was included for automated cell density measurement, ii) a H-filter was included to extract small molecules to a cell-free acceptor phase and iii) a high resolution separation was developed allowing for simultaneous monitoring of glucose, glutamine, leucine/isoleucine and lactate. Digital image processing provided an automated cell counting method with improved ease of use and higher reliability than traditional cytometry especially for cell densities above 3.5 x 10^6 cells.mL^{-1}. The H-filter allowed for the direction of particulate-free sample that contained at least 30% of the target analytes towards the analytical system without the necessity of filters or membranes that require replacement or maintenance. With the importance of glucose as a biomarker, a CE separation method was also developed using a BGE with a pH>12 to enable simultaneous monitoring of the essential metabolic biomarkers, glucose, glutamine, leucine/isoleucine and lactate. Using less than 4% of media (41 μL per run), cell density and the metabolic profile of a human lymphocyte cell line were analysed every 30 minutes over 4

days. In data processing, the correction of the chemical measurements for cell density enabled for the comparison of data between runs. Importantly, correlation of the lactate profile with cell density provided insight in the metabolic state of the cells, allowing for delineation of the lactate production as a result of metabolism and increasing cell density. Data analysis revealed a metabolic change from lactate production to lactate consumption at around 50 h, behaviour that is characteristic of fast growing cells.

Ethics and governmental regulations have led to a move away from animal models to *in vitro* methodologies for screening and testing compounds in early stage pharmacological studies. Recognising the limitation of endpoint measurements as obtained using conventional assays in terms of temporal resolution, in Ch 4 the development of a SI system for pharmacological assays is discussed. To minimize biological variability, drug assays and controls have to be conducted simultaneously from the same starting culture. For traditional assays, plate readers have replaced assays in flasks to increase the sample throughput and enable the study a range of concentrations including positive and negative controls. Because the impracticality of connecting each parallel culture to an individual SI-CE system, the focus of Ch 4 was the development of a method that allowed for monitoring of five parallel cultures using a single analytical system. Through the addition of a 7 port valve to direct sampling from five individual reactors and careful experimental design where sampling from one culture was conducted whilst the previous was analysed, an on-line, fully automated system for near real-time monitoring of simultaneous five parallel suspension cultures was developed. Like the system described in Ch 3, this system was also capable of cell density measurement and a high-resolution separations, but could do so with a 12 minute turn around time, enabling one sample per hour from each culture. Over a period of 4 days, 480 analyses were conducted, or 96 per culture. This new approach was applied to a pharmacological study investigating the metabolic effects of three pharmaceuticals, rotenone, β-lapachone and clioquinol, using

lactate as a metabolic indicator. The mitochondrial inhibitor rotenone as well as the indirect mitochondrial poison clioquinol both showed a dose-related increase in lactate production as a result of their effect on the mitochondria. Their profiles were different though, with rotenone mainly exerting its effect in the first 20 hr, whereas for clioquinol the lactate production in treated cultures only started to deviate from the control in the period between 20 hr and 40 hr. Interestingly, the lactate measurements in β-lapachone agreed with the previously reported drop in lactate supply as a result of the presence of this NAD^+ producing compound, but when corrected for cell density there was no statistical significance between the treatments and control. This suggests the previously presumed lactate production inhibiting effect of β-lapachone may have been the effect of toxicity and reduction in cell density rather than a direct metabolic effect on the cells. .

The overall aim of this thesis was to investigate the potential SI-CE for bioprocess monitoring. A review of the literature revealed a lack of adequate sampling systems hindered the use of CE for automated, online monitoring by capillary electrophoresis. In this thesis, new sampling systems were introduced for sampling from adhesion cultures (Ch 2), suspension cultures (Ch 3) and parallel suspension cultures. Combination of the sampling and sample treatment units with carefully optimized separation chemistry allowed for the demonstration of SI-CE for online monitoring of fermentations. With the majority of the work presented in this thesis focusing on the use of lactate as metabolic biomarker, it is important to realize that by changing the separation chemistry, the SI-CE systems will be directly applicable to the analysis of different analytes, showing the flexibility of the presented approach. In comparison to chromatographic techniques where typically off-line sample pre-treatment is required and separations are slow, the advantages of speed, automation and low sample consumption of the presented systems should be recognised by the biotechnologist community. The developed systems provide a promising new platform for

near real-time monitoring of chemical changes in a diverse range of adhesion and suspension cultures, potentially providing a practical way meet the future demands in process monitoring in the biopharmaceutical industry and in pharmacological and biotechnological studies.

5.1 Future directions

Further research and development is required before the developed platforms can be used by a non-expert in a routine setting. Further advances are particularly needed in the areas of integration and ease of use, increase in throughput and expansion of analyte set:

1. Integration and ease of use.

   The presented systems are predominantly assembled using Custom off the Shelf (COTS) parts, including commercially available tubing, valves, fittings and capillaries. Whilst adequate for proof of principle experiments, integration and consolidation of fluidic functionalities onto a single platform would reduce the complexity of the system, and reduce dead volume as discussed below. An obvious staring point would be to use the inlet of the H-filter for the cell density measurement, eliminating one of the microfluidic units. Recent advances in out laboratory have demonstrated the potential of computer controlled Takasago RP-Q1 peristaltic pumps for both pumping and valving, potentially eliminating the need of the use of the 6 and 7 port valves.

   The separation conditions developed for the CE analysis of glucose requires its deprotonation and hence required the use of BGE systems with pH>12. Fused silica capillaries were found to be unstable at this pH, but the PMMA capillaries also required replacement every 24 hr. The use of a less corrosive BGE would improve the lifetime of the coating and potentially avoid the need to replace the capillary. To provide the glucose with a charge required for its electrophoretic separation,

negatively charged boronate- complexes could be used in combination with a borate buffer[1].

2. Increasing throughput

   For most bioprocess monitoring, a 12 minute time interval between analyses, or 1 hr between two data points from the same reactor when 5 parallel cultures are monitored, is sufficient for most biological and pharmacological applications. When a higher throughput is required based on more stringent time requirements, for example for studying quickly acting drugs, or for monitoring more than five cultures in parallel, changes to the hardware will be required. Increasing the turn around rate to minutes and below will require a reduction of dead volume and increase in analysis time, which could be achieved by integration of all elements required for analysis onto a microfluidic device. To avoid the use of external pumps and valves, pneumatically actuated "lifting gate" microvalves and pumps as developed by Kim *et al.* could be included to enable high-efficiency integrated pumping operations [2,3].

   For pharmacological studies, integration of a chip-based SI-CE system with microfluidic microbioreactors[4] would provide the ultimate screening platform with response times in seconds. The Kennedy group has already demonstrated online electrophoretic immunoassays to study hormone excretion from isolates Islets of Langerhans using perfusate[5,6,7,8]. A similar approach could be applied without much difficulty for SI-CE of adhesion cultures, and could be used in combination with a H-filter or using inertial flow systems for suspension cultures.

3. Expansion of analyte set

   Culturing media contains a complex mixture of small and macromolecules that can be anionic, cationic or neutral. In combination with the limited peak capacity especially when moving from long fused silica capillaries to separation channels on microchips,

it will not be feasible to separate all analytes of interest using a single separation system. By replacing the T-piece connecting the separation capillary and waste with a cross piece, Gaudry *et al.*, demonstrated the simultaneous analysis of anions and cations in individual capillaries by SI-CE[9]. Taking advantage of enhanced fluidic control on microfluidic devices, a novel hydrodynamic "split injection" was developed on a PMMA device exploiting laminar flow to use individualized separation chemistries for the cation and anion separation[10]. Three cations and three anions were separated simultaneously and a series of 100 sequential injections has given excellent reproducibility requiring only 70.5 seconds per analysis. For bioprocess monitoring, the capillary systems presented in this thesis could be expanded for simultaneous cation and anion analysis by replacing the T-piece with a cross piece. Recognising optimizing the separation chemistry for a sample as complex as media for both cation and anion separations will be a challenge, this would allow a significant increase in the number of analytes that can be investigated without increasing the sample consumption. Some more complex fluidic control and one or more crosses would allow the injection from the same sample flow into separation chemistries dedicated to peptide or protein separations, or maybe include a micellar system for the analysis of neutrals. Using this approach, the minimal increase in sample consumption would simplify the BGE selection process for each individual separation. Practically, the fluidic complexity of outscaling will be easier to manage on a microfluidic platform using external pumps and/or pneumatically controlled pumps and valves.

5.2 Reference

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